Pharmacological Characterisation of the Human 5-HT_{1A} receptor and its Inhibitory G protein Fusions

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By

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Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
AppNHp	βγ-imidoadenosine 5'-triphosphate
ATP	5' adenosine triphosphate
B_{max}	expression level
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cAMP	adenosine 3':5'-monophosphate
CCV	clathrin coated vesicle
cDNA	complementary deoxy ribonucleic acid
cpm	counts per minute
C terminal	carboxyl/COOH terminal
Da	Dalton
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DRY	Asp-Arg-Tyr
DTT	dithiothreitol
EC_{50}	effective concentration 50%
ECL	enhanced chemiluminescence
EDTA	diaminoethanetetra-acetic acid disodium salt
ERK	extracellular signal regulated kinase
G protein	guanine nucleotide binding regulatory protein
GAP	GTPase activating protein
GABA	γ-amino butyric acid
GDP	guanosine diphosphate
G_i	G protein originally characterised by its ability to inhibit adenylyl cyclase activity
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
G_s	G protein originally characterised by its ability to stimulate adenylyl cyclase activity
GST	glutathione-S-transferase

GTP	guanosine triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
HEK293	human embryonic kidney 293
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
5-HT	5-hydroxytryptamine
$5\text{-HT}_{1A}/G_{i1\alpha}$	5-HT_{1A} receptor fused to the $G_{i1\alpha}$ protein
$5\text{-HT}_{1A}/G_{i1\alpha}C^{351}G$	5-HT_{1A} receptor fused to the $G_{i1\alpha}Cys^{351}Gly$ mutant protein
$5\text{-HT}_{1A}/G_{i1\alpha}C^{351}I$	5-HT_{1A} receptor fused to the $G_{i1\alpha}Cys^{351}Ile$ mutant protein
$5\text{-HT}_{1A}/G_{o1\alpha}$	5-HT_{1A} receptor fused to the $G_{o1\alpha}$ protein
$5\text{-HT}_{1A}/G_{o1\alpha}C^{351}G$	5-HT_{1A} receptor fused to the $G_{o1\alpha}Cys^{351}Gly$ mutant protein
$5\text{-HT}_{1A}/G_{o1\alpha}C^{351}I$	5-HT_{1A} receptor fused to the $G_{o1\square}Cys^{351}Ile$ mutant protein
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	inhibition concentration 50%
IPTG	isopropyl-β-D-thiogalactoside
IP_3	inositol 1,4,5 trisphosphate
JNK	c-Jun N-terminal kinase
K^+	potassium ion
K_d	concentration of ligand that will bind to half the receptors at equilibrium
K_i	affinity of the receptors for the competing drug
K_m	affinity of an enzyme for substrate
LSD	lysergic acid diethylamide
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
MPPF	4-(2'-methoxy)-phenyl-1-[2'-(N-2"-pyridinyl)-p-fluorobenzamido]ethyl-piperazine
mRNA	messenger ribonucleic acid
N-terminal	amino/NH_2-terminal
PBS	phosphate buffered saline
PH	pleckstrin homology
PKA	protein kinase A
PKC	protein kinase C

PLC	phospholipase C
pK_i	negative log of the K_i
PMA	phorbol 12-myristate 13-acetate
ptox	pertussis toxin
R	inactive receptor form
R*	active receptor form
RGS	regulator of G protein signalling
SD	standard deviation
SE	standard error
SERT	serotonin transporter
SDS	sodium dodecylsulfate
SSRI	serotonin selective re-uptake inhibitor
TAE	tris-acetate-EDTA
TM	transmembrane
V_{max}	maximum reaction rate
WAY100635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl[ethyl]-N-2-pyridinyl-cyclohexane-carboxamide
WT	wild type

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Abstract

Fusion proteins between the human 5-HT_{1A} receptor and wild type and pertussis toxin resistant forms of the $G_{i1\alpha}$ and $G_{o1\alpha}$ proteins were constructed and stably expressed in HEK293 cells. These constructs were assessed in terms of second messenger system modulation, receptor-G protein affinity and high affinity GTPase activity and the effects that RGS proteins had on this.

In an intact cell assay 5-HT activation of the 5-HT_{1A} receptor-G protein fusions concentration dependently inhibited forskolin stimulated adenylyl cyclase activation. Treatment with pertussis toxin abolished 5-HT_{1A} receptor mediated effects for the receptor alone and the wild type G protein fusions but did not have any effect on cells expressing either the Gly^{351} or Ile^{351} $G_{i1\alpha}$ and $G_{o1\alpha}$ 5-HT_{1A} receptor-G protein fusion constructs, a result that indicated a lack of coupling to the endogenous pool of G protein by the fusion proteins. Consistent with the results of Bahia *et al.* (1998) the concentration of 5-HT required to produce a 50% reduction in adenylyl cyclase activity was lowest for the Ile^{351} G_{α} protein mutant followed by the WT G_{α} proteins and was highest for the Gly^{351} G_{α} protein mutants. Although not significant the results, consistent with the literature, also indicated that a lower concentration of 5-HT was required to produce a 50% reduction in adenylyl cyclase activity for the 5-HT_{1A} receptor-$G_{i1\alpha}$ than for the $G_{o1\alpha}$ fusion proteins.

The ability of GDP and suramin to prevent [^{3}H]-8-OH-DPAT specific binding to membranes expressing the 5-HT_{1A} receptor and each of the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins was assessed in radioligand binding assays. The concentrations of either GDP or suramin required to reduce specific binding by 50% were significantly lower for the Ile^{351} mutants than for the wild type proteins with the Gly^{351} mutant fusion proteins requiring the highest concentrations. This provided an indication of the affinity of the 5-HT_{1A} receptor for the various G proteins and followed the trend that the more hydrophobic the residue at position 351 of a $G_{i\alpha}$ and $G_{o\alpha}$ proteins the higher the affinity of the 5-HT_{1A} receptor G protein interaction. The results also indicated that the 5-HT_{1A} receptor had a higher affinity for $G_{i1\alpha}$ than $G_{o1\alpha}$ protein interactions. 8-OH-DPAT competition for [^{3}H]-MPPF specific binding to membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}$G/I fusion proteins indicated that a significantly greater proportion of the Ile^{351} mutant G proteins exist in a 5-HT_{1A} receptor coupled form than the Gly^{351} G protein mutants.

The high affinity GTPase activity of membranes expressing the fusion proteins and the effects of a range of purified RGS protein concentrations was examined. While agonist stimulation of GTPase activity produced an increase in V_{max} it did not effect the K_m for GTP. The addition of either RGS1 or RGS16 proteins however produced concentration dependent increases in both the V_{max} and the K_m due to their GAP activity at the $G_{i1\alpha}$ and $G_{o1\alpha}$ proteins. The effects of the two RGS proteins were not significantly different, but both proteins produced greater increases in GTPase activity in membranes expressing the $G_{o1\alpha}$ fusion protein than membranes expressing the $G_{i1\alpha}$ fusion protein. Neither RGS protein had any significant effect on basal GTPase activity for membranes expressing the Gly^{351} mutant fusion proteins. Both RGS1 and RGS16 proteins did however produce concentration dependent increases in basal GTPase activity in membranes expressing the Ile^{351} fusion proteins, a result that reflects the greater intrinsic constitutive GTPase activity of the 5-HT_{1A} receptor-Ile^{351} G protein fusion constructs.

Taking advantage of this latter result, the effects of a range of ligands on high affinity GTPase activity in the absence and presence of a maximal concentration of RGS1 protein were examined. In the absence of RGS1 protein WAY100635 acted as a weak partial agonist with spiperone, methiothepine, (+)-butaclamol, chlorpromazine and thioridazine acting as inverse agonists. In the presence of RGS1 protein, the increase in basal GTPase activity for the $G_{i1\alpha}C^{351}I$ and $G_{o1\alpha}C^{351}I$ fusion proteins of 165% and 400% respectively allowed for accurate quantification of ligand effects. Only haloperidol was found to be a neutral ligand at the 5-HT_{1A} receptor-G protein fusions in this assay. Thus, the use of constitutively active receptor-G protein fusions and RGS proteins in a high affinity GTPase assay allowed for accurate discrimination between weak partial, neutral and inverse agonist activity.

Chapter 1

Introduction

1 Introduction

1.1 Serotonin History

The discovery of serotonin was made by an Italian scientist, Dr. Vitorio Erspamer, working for the Institute of Comparative Anatomy and Physiology in the University of Pavia, Italy. His work on the smooth muscle constricting and contracting properties of various amine substances lead him to isolate a substance from the enterochromaffin cells of the rabbit gut. This substance caused smooth muscle contraction, especially in the rat uterus, and tests revealed that it was most likely an indole. He named the substance enteramine (Erspamer and Vialli, 1937) and published further on its properties (Erspamer, 1946, Erspamer and Ghiretti, 1951; Erspamer, 1948; Erspamer and Boretti, 1951). It was not until 1952, when it was established that Dr. Erspamer had been working on the same substance that Irvine Page, Maurice Rapport and Arda Green had been researching since 1948, that enteramine became know as serotonin (Erspamer and Asero, 1952).

Irvine Page worked on the etiology and treatment of essential hypertension at the Cleveland Clinic in 1945. Dr. Page was looking for endogenous substances in blood that would lead to vessel constriction and during this research came across a substance which he decided must be removed from the blood before any further work could be done on the hypertension-producing factor. To this end he recruited Maurice Rapport and Arda Green who succeeded in isolating and characterising the substance, naming it serotonin. Arda Green is also known for the first isolation of luciferase in 1953 from fireflies. Isolation of 5-HT was not an easy process and required over seven thousand litres of blood from the Cleveland slaughterhouse resulting in the purification of a very small quantity of serotonin. The first publication describing the partial purification of serotonin was in *The Journal of Biological Chemistry* in 1948 (Rapport *et al.*, 1948a). Dr. Rapport continued his work on serotonin and completed the isolation later that year (Rapport *et al.*, 1948b). Continuing his work at Colombia University Dr. Rapport published the chemical structure of serotonin (Rapport, 1949) and following a collaboration with Upjohn Pharmaceutical and Abbott Laboratories synthetic serotonin was made available for research.

The presence of serotonin in the brain was reported by Betty Mack Twarog in 1953 (Twarog and Page, 1953). Dr. Twarog had previously worked on the mussel, *Mytilus edulis* and the neurotransmitters responsible for a phenomenon called "catch" where the molluscs' muscles remained contracted and resisted stretch long after the period of excitation had passed. By 1951 Dr. Twarog had identified the contracting neurotransmitter as acetylcholine, but had not identified the relaxing neurotransmitter. Upon reading papers from Dr. Erspamer on enteramine, its isolation from octopus salivary glands, its excitation of mollusc heart, and the revelation that enteramine and serotonin were one and the same, Dr. Twarog became convinced, and subsequently demonstrated that the relaxant neurotransmitter was serotonin (Twarog, 1954). Dr. Twarog moved to the Cleveland Clinic in late 1952 and proceeded to demonstrate for the first time, despite the doubts of Dr. Page, the presence of serotonin in mammalian brain (Twarog and Page, 1953).

The final person involved in establishing serotonin as one of the most important neurotransmitters involved in mental illness was Dilworth Wayne Woolley. Dr. Woolley had developed a theory in which specific substances that were structurally related to naturally occurring metabolites could interfere with the functioning of those metabolites. He referred to the synthetic compounds as "antimetabolites". Dr. Woolley joined the Rockerfeller University in 1939 and despite being completely blind due to severe diabetes, "saw" the structure of serotonin in that of LSD. He demonstrated that LSD could block the effects of serotonin in the rat uterus, and these effects could be mimicked by bufotenine. He also believed that serotonin had a role in brain development and first published on his hypothesis in 1954 (Woolley and Shaw, 1954). Unable to get his work and hypotheses published in *Lancet*, Wolley published a book on them in 1963, *The Biochemical Bases of Psychoses or the Serotonin Hypothesis about Mental Illness* (Wolley, 1963). Dr. Woolley was elected member of The National Academy of Sciences and The National Academy of Arts and Sciences in recognition of his work.

Following this groundbreaking work, using Falck-Hillarp histochemical fluorescence, 5-HT neurons and their projections were described (Dahlstrom and Fuxe, 1964; Fuxe, 1965). Today it is an established fact that no region of the mammalian CNS lacks 5-HT innervation (Dahlstrom and Fuxe, 1964; Steinbusch, 1981, 1984; Azmitia, 1986; Jacobs and Azmitia, 1992; Azmitia-Whitaker and Azmitia, 1995).

1.2 Serotonin Synthesis, Metabolism and Regulation

Serotonin is synthesised in neurons from the amino acid (L)-tryptophan in two steps (Figure 1.1, A). Firstly, catalysed by tryptophan hydroxylase with the cofactor tetrahydrobiopterin, L-tryptophan is converted to L-5-hyrdroxytryptophan. This step is followed by aromatic L-amino acid decarboxylase catalysing conversion, in the presence of the cofactor pyridoxal phosphate, to 5-hydroxytryptamine (5-HT or serotonin). The degradation of 5-HT is also a two step process (Figure 1.1, B). Initially, the mitochondrial enzyme monoamine oxidase (MAO) with the cofactor flavin adenine dinucleotide (FAD), catalyses conversion to 5-hydroxyindoleacetaldehyde. This intermediate product is then rapidly converted to 5-hydroxyindolacetic acid (5-HIAA) by aldehyde dehydrogenase.

Neurotransmitter release from neuronal cells by exocytosis is in response to depolarisation. Depolarisation leads to the opening of voltage sensitive calcium channels resulting in calcium entry, the major signal that links depolarisation and exocytosis (Burgoyne and Cheek, 1995). Secretory vesicles containing neurotransmitters that are already docked at the plasma membrane release their contents into the synaptic cleft by formation of a fusion pore, a process involving a number of calcium sensitive proteins (Augustine *et al.*, 1985; Smith and Augustine, 1988; Leveque *et al.*, 1992). The amount of neurotransmitter released is subject to receptor-dependent regulation with the main autoregulatory effect of 5-HT on its own release being inhibitory.

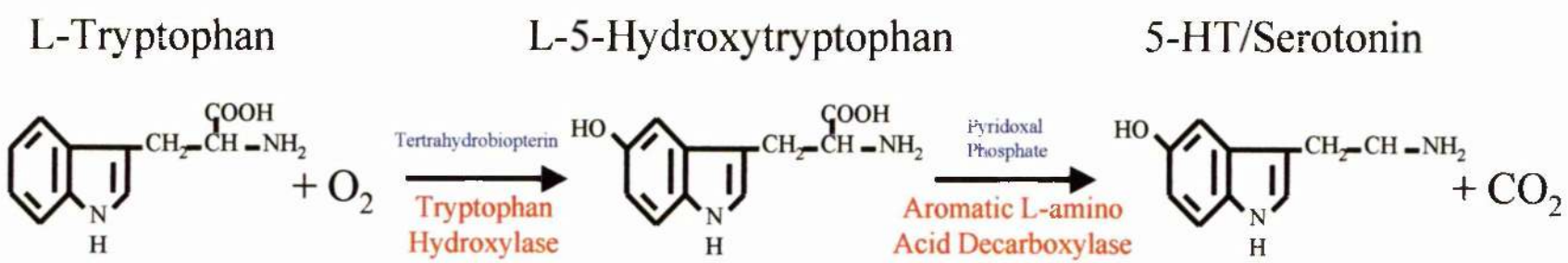
Serotonin acting at the 5-HT_{1A} receptor has been shown to inhibit 5-HT release by reducing high threshold Ca^{2+} currents (Penington and Kelly, 1990; Penington *et al.*, 1991; Penington and Fox, 1994) and increasing conductance to K^+ (Aghajanian and Lakoski, 1984; Yoshimura and Higashi, 1985). These experiments have been performed in the cell body and there is no direct electrophysiological information on the mechanism by which 5-HT regulates its own release. The receptor involved in 5-HT inhibition of its own release is dependent on the area of the neuron examined. As well as regulation by the 5-HT_{1A} autoreceptor in somatodendrons, release is controlled by terminal 5-$HT_{1B/1D}$ autoreceptors (Starke *et al.*, 1989). The 5-$HT_{1B/1D}$ receptors can alter 5-HT release without effecting 5-HT neuron firing activity (Crespi *et al.*, 1990). There is now evidence that at the cell body, inhibition of 5-HT release

Figure 1.1 Synthesis and metabolism of 5-hydroxytryptamine

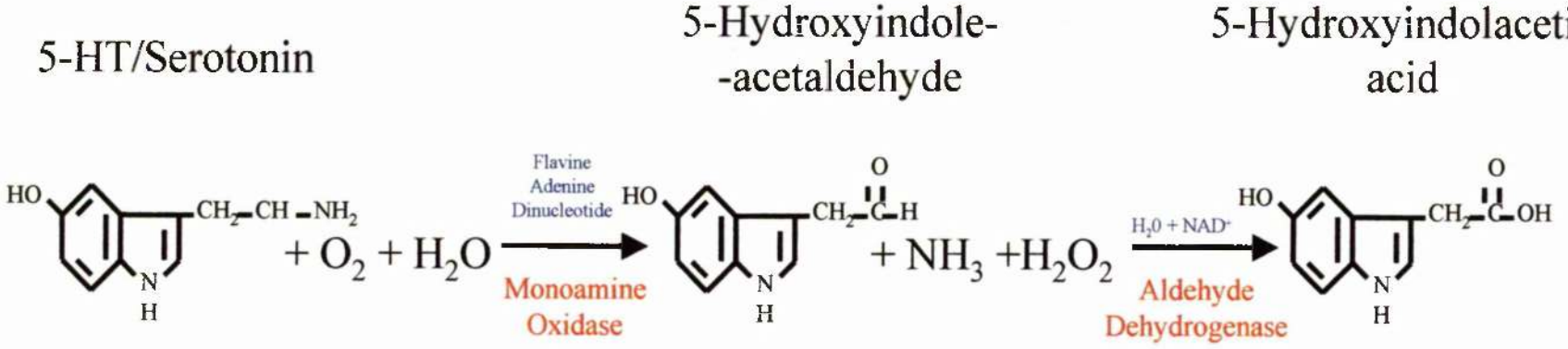
Panel A: The synthesis of 5-hydroxytryptamine from the amino acid L-tryptophan.

Panel B: The metabolism of 5-hydroxytryptamine to 5-hydroxyindolacetic acid.

Panel A



Panel B



may not only be regulated by the 5-HT_{1A} receptor, because the non 5-HT_{1A} agonist mianserin blocked decreases in 5-HT release stimulated by the 5-HT_1 agonist RU 24969, but WAY100135 did not (Pineyro *et al.*, 1995, 1996). Significantly, a decrease in 5-HT neuron firing was also not recorded.

As well as autoregulatory mechanisms controlling the release of 5-HT there are mechanisms that indicate the involvement of other receptors. A decrease in 5-HT release is measured in the dorsal raphe nuclei by either systemic or intraraphe administration of GABA or GABA agonists (Scatton *et al.*, 1985; Becquet *et al.*, 1990). It has also been found that activation of presynaptic κ opioid receptors located on the glutamatergic fibres in midbrain slices reduces excitatory postsynaptic potentials due to electrically evoked release of excitatory amino acids from afferent fibres onto 5-HT neurons (Pinnock, 1992). GABA and glutamate have been shown to modulate 5-HT release in rostral rhombencephalic raphe cells in primary culture (Becquet *et al.*, 1993b). GABA produces its negative modulation mainly via the $GABA_A$ and $GABA_B$ receptors, and EAA induced 5-HT release through NMDA receptors (Becquet *et al.*, 1993a). The D_2 dopamine receptor has been indicated also in the regulation of 5-HT release (Ferre and Artigas, 1993; Ferre *et al.*, 1994).

Following its release into the synaptic cleft, 5-HT is actively removed by a high affinity transporter located on presynaptic neuronal membranes (Kuhar, *et al.*, 1972; Kanner and Schuldiner, 1987; O'Reilly and Reith, 1988). The transporter, SERT, functions in series with the vesicular transporter that sequesters 5-HT into secretory vesicles. SERT is an integral membrane protein with twelve transmembrane spanning domains coupling to the uptake of Na^+ and release of Cl^-. Purified functional SERT has a molecular weight of 300,000 (Ramamoorthy *et al.*, 1993), and cloned SERT 70,000 (Blakely *et al.*, 1991; Lesch *et al.*, 1993; Ramamoorthy *et al.*, 1993), leading to the proposal that SERT may exist as a homotetramer (Ramamoorthy *et al.*, 1993). However, Chang and co-workers demonstrated that dimeric and monomeric transporter activity was similar, with tetrameric transporters having substantially less activity (Chang *et al.*, 1994).

The mechanism of 5-HT uptake by SERT has been characterised in a number of preparations, including platelets (Rudnick, 1977; Nelson and Rudnick, 1979, 1982) and mouse brain plasma vesicles (O'Reilly and Reith, 1988; Reith *et al.*, 1989) and stable expression in different systems (Blakely *et al.*, 1991; Hoffman *et al.*, 1991; Corey *et al.*, 1994, Gu *et al.*, 1994). An inward Na^+ concentration gradient has

been demonstrated to be required for 5-HT uptake. External Na^+ increases the V_{max} and decreases K_m for 5-HT (Cool *et al.*, 1990). Stoichiometry between 5-HT, Na^+ and Cl^- has been shown to have a 1: 1: 1 ratio (Cool *et al.*, 1990; Talvenheimo *et al.*, 1983; Gu *et al.*, 1994; Nelson and Rudnick, 1982).

1.3 G protein-Coupled Receptors

For a large number of both natural and synthetic compounds, transduction of signals from the extracellular to the intracellular environment is via cell membrane bound receptors. Heterotrimeric GTP-binding protein (G protein) coupled receptors (GPCRs) are the largest class of membrane bound receptors and are found in eukaryotes and some prokaryotes. The family has an estimated 1100 members in *Caenorhabditis elegans*, comprising 5% of its genome (Bargmann, 1998), at least 160 members in Drosophila, and is estimated to comprise at least 700 members in the human genome (Miklos *et al.*, 2000). The presence of GPCRs in plants (Plakidou-Dymock *et al.*, 1998), yeast (Dohlman *et al.*, 1991), the slime mold *Dictyostelium discoideum* (Devreotes, 1994), and in protozoa and metazoa (Vernier *et al.*, 1995; New and Wong, 1998) indicates that they may be one of the oldest families of proteins devoted to signal transduction.

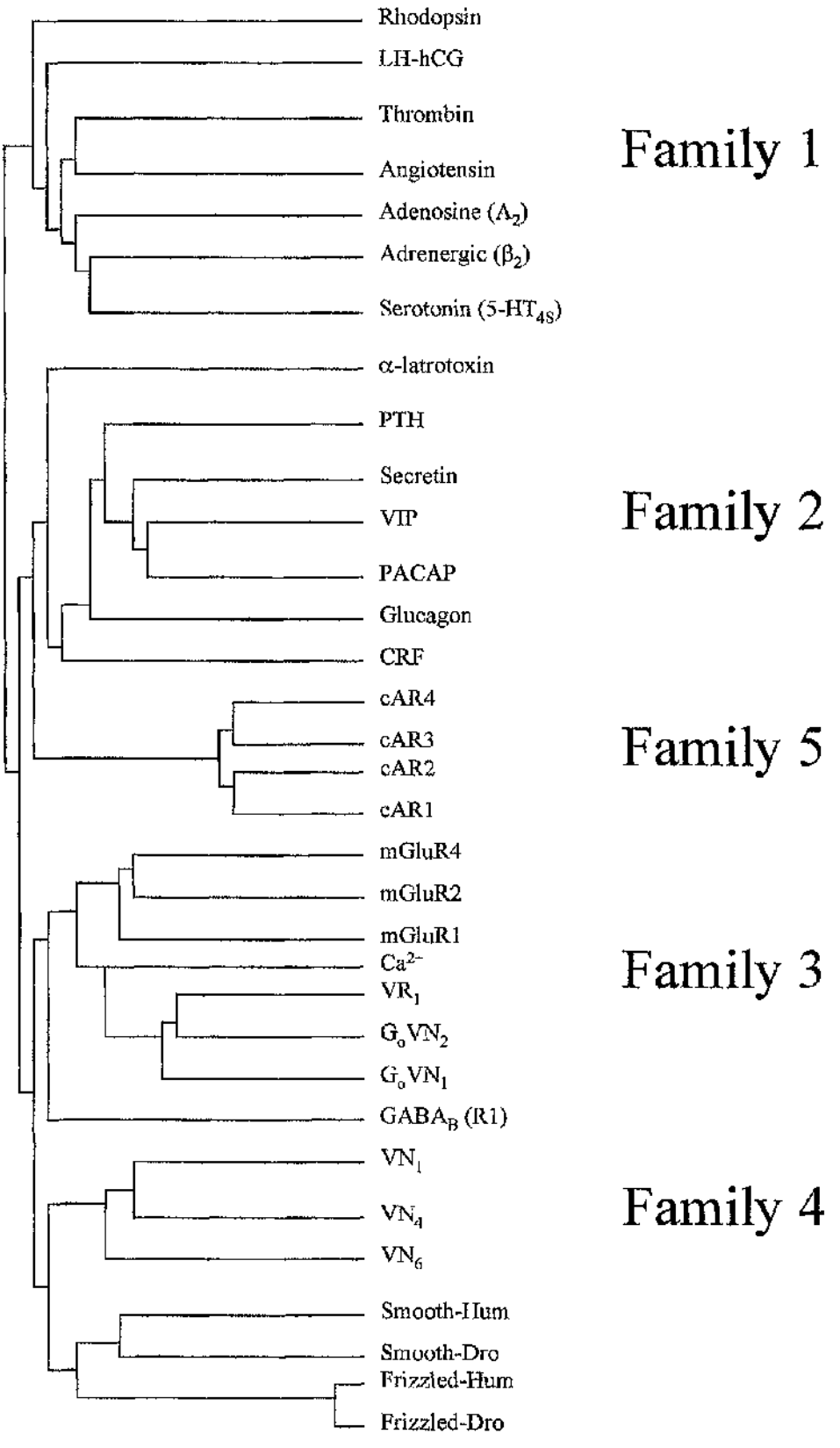
The GPCR family of cell surface receptors transduces signals from a wide variety of extracellular stimuli, such as nucleotides, peptides, amines, odorants, Ca^{2+} ions and photons. They regulate the activity of ion channels, enzymes and transport vesicles by promoting the exchange of GTP for GDP, activating the various classes of G proteins and leading to variations in an enormous range of cell functions (Gilman, 1987).

GPCRs can be classified into a number of families (Figure 1.2). Families 1, 2 and 3 contain the known human receptors, with two additional families encompassing the fungal pheromone and *Dictyostelium* GPCRs. These families are divided further into subclasses that are defined by sequence similarity, ligand binding properties and functional domains (Horn *et al.*, 1998; Bockaert and Pin, 1999).

Family 1 contains most GPCRs, including the odorant receptors, and sequence alignment of receptors in this class shows approximately 20 conserved amino acids. These include two cysteine (Cys) residues in extracellular loops 1 and 2 that form a di - sulphide bridge necessary for maintaining correct receptor

Figure 1.2 Classification of GPCRs

The diverse nature of the GPCR superfamily allows only for general classification into five families (1-5). The first three families contain the human GPCRs with the vast majority fitting into family 1, a family that encompasses GPCRs for small ligands, e.g. β-adrenergic receptors (family 1a), for peptides (family 1b), and for glycoproteins (family 1c). Family 2 have a similar morphology to family 1c, but share no sequence homology and family 3 contain the metabotropic glutamate and Ca^{2+} sensing receptors. Family 4 contains the pheromone receptors and lastly family 5 the cAMP receptors found in *Dictyostelium.* A further family containing the "frizzled" and "smoothened" embryonic receptors has been proposed. Adapted from Bockaert and Pin, 1999.


Rhodopsin
LH-hCG
Thrombin
Angiotensin
Adenosine (A_2)
Adrenergic (β_2)
Serotonin (5-HT_{4S})
α-latrotoxin
PTH
Secretin
VIP
PACAP
Glucagon
CRF
cAR4
cAR3
cAR2
cAR1
mGluR4
mGluR2
mGluR1
Ca^{2-}
VR_1
G_oVN_2
G_oVN_1
$GABA_B$ (R1)
VN_1
VN_4
VN_6
Smooth-Hum
Smooth-Dro
Frizzled-Hum
Frizzled-Dro
Family 1
Family 2
Family 5
Family 3
Family 4

conformation, the DRY (Asp-Arg-Tyr) motif in the proximal region of intracellular loop 2, an Asn-Pro-X-X Tyr motif in transmembrane domain 7 and a cysteine residue in the C terminal domain that can be palmitoylated generating a fourth intracellular loop. Family 1 is further divided into three subclasses. Class 1a includes the receptors for small ligands such as photons and biogenic amines, and includes the rhodopsin, β-adrenergic receptors and serotonin receptors. This subclass of GPCRs has a ligand-binding site located within the transmembrane spanning domains. Class 1b includes receptors that bind peptides such as chemokines to the N terminal region, the extracellular loops and the upper sections of the transmembrane domains. Class 1c contains receptors for glycoprotein hormones such as luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone and is characterised by large extracellular N termini involved in ligand binding that must also include binding to at least one of extracellular loops 1 or 3 (Bockaert and Pin, 1999).

Family 2 GPCRs have a similar morphology to the family 1c receptors but do not share any sequence homology and represent the second largest group of GPCRs. High molecular weight peptides such as glucagon, secretin, VIP-PACAP and calcitonin bind to this family of receptors, as does the black widow spider toxin α-latrotoxin (Krasnoperov *et al.*, 1997; Davletov *et al.*, 1998). The receptors have long N-terminal regions (>100 amino acids) that are involved in ligand binding and contain six conserved Cys residues. They also have two conserved Cys residues in extracellular loops 1 and 2 and approximately 15 other residues that are conserved in all members of this class.

The third and smallest family of GPCRs contains the metabotropic glutamate receptors and the Ca^{2+} sensitive receptors (CaR) (Pin and Bockaert, 1995). This family also contains the $GABA_B$ receptors (Kaupmann *et al.*, 1997) and a group of putative pheromone receptors coupled to the G_o protein, termed VRs and G_o-VN (Bargmann, 1997). They all possess extremely long N terminal regions that are involved in ligand binding and several conserved Cys residues in the transmembrane spanning and extracellular regions.

The fourth family comprises of the pheromone receptors (VN's) which bind to the G_i class of G protein (Dulac and Axel, 1995), and the fifth family the cAMP receptors (cAR) only found in *Dictyostelium* with as yet no established vertebrate equivalent. One final group of receptors that may constitute a family in its own right

is the "frizzled" and "smoothened" (Smo) receptors that are involved in embryonic development.

1.3.1 GPCR Structural Features

Alignment of the receptors from the various families of GPCRs shows little or no sequence homology, but all GPCRs share a common structure. They are predicted to contain seven transmembrane spanning domains, consisting of 20-30 amino acids each, linked by three extracellular and three intracellular domains of varying lengths, an extracellular N terminal region with N-glycosylation sites and an intracellular C terminal region that generally contains phosphorylation sites and may contain palmitoylation sites (Ji *et al.*, 1998; Henderson *et al.*, 1990; Dixon *et al.*, 1986). Variability in the sequence and size of these domains results in the individual ligand binding and G protein coupling characteristics of various GPCRs.

The N terminal region of GPCRs is an extracellular domain that varies in size from less than 10 amino acids for the A_{2A} (Furlong *et al.*, 1992) and A_{2B} (Pierce *et al.*, 1992) adenosine receptors to between 340 and 450 amino acids for mature glycoprotein hormone receptors (Dias *et al.*, 1992). This region plays a role in the trafficking of the receptor to the plasma membrane and contains asparagine residues within motifs for N-glycosylation (Petaja-Repo *et al.*, 2000; George *et al.*, 1986; Hughes *et al.*, 1997), and cysteine residues involved in protein folding (Green *et al.*, 1994). Work on N-glycosylation of the δ-opioid receptor demonstrated that this process is initiated co-translationally in the endoplasmic reticulum and completed in the trans-golgi network (Petaja-Repo *et al.*, 2000). It was suggested that less than 50% of synthesised receptors are fully processed and of these only a fraction make it to the surface. A rate limiting step in this process was the exit of a fully processed GPCR from the endoplasmic reticulum indicating that in part at least, N-glycosylation is a key event in the control of GPCR expression. As was indicated in the classification of GPCRs the N terminal region of all families apart from the 1a class of GPCR is involved in ligand binding. In the calcium sensing receptor (CaR), the $Lys^{47}Asp$ mutation was found to result in hypocalcemia by preventing ligand binding (Okazaki *et al.*, 1999).

The next common structural feature for GPCRs is the seven transmembrane spanning domain regions, linked by three extracellular and three intracellular loops

(Henderson *et al.*, 1990; Dixon *et al.*, 1986). The seven helices are thought to be arranged as a tight ring shaped core (Baldwin, 1993; Ji *et al.*, 1998) with the hydrophobic amino acid residues facing the lipid bilayer and the more hydrophilic residues the core. A low resolution (9Å) electron diffraction structure of rhodopsin revealed the orientation of the transmembrane α-helices (Unger *et al.*, 1997). A high resolution (2.8 Å) electron diffraction structure of rhodopsin has been published (Palczewski *et al.*, 2000) that indicated the importance of clusters of residues in maximum wavelength absorption, colour discrimination and interactions between the membrane helices and cytoplasmic surface where G protein activation occurs.

The transmembrane domains contain several highly conserved Pro residues important for receptor folding and expression. A mutagenesis study in the muscarinic M_3 receptor in which three conserved Pro residues (Pro^{242} of TM5, Pro^{505} of TM6 and Pro^{540} of TM7) were changed to Ala demonstrated a 35 to 100 fold lower expression level compared to WT receptor (Wess *et al.*, 1993). Another study on the vasopressin V_2 receptor mutated Lys^{44} of TM1 to Pro, leading to a lack of glycosylation and retention in the pre-golgi compartment. The introduction of Pro was essential as mutation to Phe led to correct processing of the receptor, although ligand binding was affected (Tsukaguchi *et al.*, 1995).

Using site directed mutagenesis of the melanocortin1 receptor, a number of important acidic residues in TM2 and TM3 in the transmembrane spanning domains involved in ligand binding have been established (Yang *et al.*, 1997). There are also a number of aromatic residues in TM's 4, 5, and 6 whose involvement has been indicated in the formation of a hydrophobic pocket for ligand binding. A naturally occurring mutation in the dopamine D_4 receptor, $Val^{194}Gly$ in TM4, that is one position removed from a Ser residue important in dopamine binding, results in a 2 fold decrease in binding of dopamine, clozapine and olanzapine over the WT receptor (Liu *et al.*, 1996).

Studies on GPCRs such as the rhodopsin receptor revealed that a change in the relative orientation of TM3 and TM6, with a rotation of TM6 and a separation from TM3, is associated with the switch from the inactive to active conformation and the unmasking of the G protein binding site (Farrens *et al.*, 1996; Bourne *et al.*, 1997; Javitch *et al.*, 1997). This change in conformation effects the orientation of intracellular loops 2 and 3 (directly linked to TM3 and TM6 respectively) effecting

what constitutes one of the key sites involved in G protein recognition and activation (Spengler *et al.*, 1993; Pin and Bockaert, 1995; Wess, 1997).

The N and C terminal regions of the third intracellular loop have been implicated in specificity for and coupling of G proteins (Cotecchia *et al.*, 1992; D'Angelo *et al.*, 1996; Chung *et al.*, 1999), with roles also established for intracellular loops 1 and 2 (Wess, 1998). The intracellular loops are involved with interactions with other cellular proteins such as the β-arrestins (Mukherjee *et al.*, 1999) and contain phosphorylation sequences for G protein receptor kinases (Tsuga *et al.*, 1998) and second messenger kinases (Yuan *et al.*, 1994; Hipkin *et al.*, 2000), all of which are involved in receptor desensitisation.

Contrary to previous beliefs that GPCRs required agonist binding to generate a second messenger response, it is now well established that GPCRs can signal in the absence of ligand. This intrinsic constitutive activity was described initially for the δ-opioid receptor endogenously expressed in NG108-15 cells (Costa and Herz, 1989), but only accepted widely after reports of intrinsic constitutive activity in overexpressed mutant α_{1B} and β_2 receptors (Lefkowitz *et al.*, 1993). Studies using CAM (Constitutively Active Mutant), WT and inactive α_{1B} receptors have indicated that Arg^{143} in the highly conserved DRY motif at the cytosolic end of TM3 plays a significant role in determining GPCR activation state (Scheer *et al.*, 1996). Mutation to Ala or Ile resulted in a complete loss of receptor mediated response but mutation to Lys conferred constitutive activity. It was suggested that hydrogen bonds between various residues form a hydrophobic pocket for Arg^{143} resulting in several residues in the second and third intracellular loops not being exposed to the cytosol. Following agonist binding or in the CAM structures Arg^{143} moves out of this hydrophobic pocket and exposes the hidden residues. In an oversimplified model, a GPCR is in a state of equilibrium between an inactive R form and an active R* form. In the absence of agonist or CAM mutations the equilibrium favours the R form, but following agonist binding or CAM mutation of the GPCR, the equilibrium favours the R* form.

The Asp^{142} in the DRY motif of the α_{1b} adrenoceptor also has an indicated role in the interaction with GRKs and β-arrestins (Mhaouty-Kodja *et al.*, 1999). It was speculated that the residue either is directly involved in the binding of these

proteins or that its mutation results in conformational changes in intracellular loops 2 and 3 so that they can no longer interact with the regulatory proteins.

CAM GPCRs can be generated by mutations in areas other than the DRY motif. Mutation of Thr^{373} (e.g. to either Cys or Lys) in the C terminal portion of the third intracellular loop of the α_2 adrenergic receptor generates basal levels of activity approaching those of agonist stimulated levels in WT receptors (Ren *et al.*, 1993). Also, more simply, constitutive levels of activity can be generated by overexpression of WT receptors (Chidiac *et al.*, 1994; MacEwan and Milligan, 1996; Smit *et al.*, 1996). This leads to an increase in the absolute amount of R* present.

On the extracellular loops, the single most important conserved amino acids are two Cys residues, one in extracellular loop 1 and one in loop 2, which form a disulphide bond and play a role in the receptor conformation (Green *et al.*, 1994). An additional disulphide bridge has been found between a Cys in extracellular loop 3 and the N terminal domain in, for example, the angiotensin receptor.

The transmembrane spanning domains, which play a significant role in ligand binding and receptor activity, are linked finally to the GPCR's C terminal tail. The C terminal tails of GPCRs contain Ser and/or Thr residues that are targeted for phosphorylation by G protein receptor kinases (GRKs) and are involved in receptor desensitisation (Bouvier *et al.*, 1988; Seibold *et al.*, 1998). Following phosphorylation, GPCRs interact with a class of proteins called arrestins (Pippig *et al.*, 1993; Freedman *et al.*, 1995). These proteins target GPCRs for internalisation via clathrin coated vesicles but also have been shown to couple GPCRs to the activation of Src-like kinases and facilitate formation of complexes including components of the MAPK and JNK pathways (McDonald *et al.*, 2000; van Biesen *et al.*, 1996).

A number of GPCRs contain a Cys residue in the C terminal domain which serves as a site for palmitoylation (O'Dowd, 1989; Ovchinnikov *et al.*, 1998), that in many cases forms a fourth intracellular loop when the palmitate inserts into the plasma membrane and has been shown to affect G protein interaction (Milligan *et al.*, 1995). Palmitoylation at these Cys residues may also have a role in receptor turnover, expression and localisation (Kennedy and Limbird, 1993; Eason et al., 1994). The tail may contain domains that allow interaction with a variety of other proteins which can mediate GPCR signalling, such as Homer/Vesl proteins (Brakeman *et al.*, 1997), and calcyon (Lezcano *et al.*, 2000). The β_2-adrenergic receptor has been shown to interact via a PDZ domain in its C terminal tail with the Na^+/H^+ exchanger regulatory

factor (NHERF) in an agonist dependent fashion (Hall *et al.*, 1998). The rhodopsin receptor has been shown to interact with InaD (Chevesich *et al.*, 1997; Xu *et al.*, 1998), a multi PDZ domain containing scaffolding protein which interacts with signalling proteins such as phospholipase Cβ, protein kinase C and the TRP ion channel (Huber *et al.*, 1996; Shieh and Zhu, 1996; Chevesich *et al.*, 1997; Tsunoda *et al.*, 1997; Xu *et al.*, 1998).

1.3.2 GPCR Activation States

The activation state of a GPCR is affected by a large number of factors, including the level of G protein expression and most obviously by agonists, the binding of which can be inhibited by antagonists. Antagonists may bind to the receptor at the same site as agonists, but do not effect the activation state. Agonists can be divided into three classes, full, that bind to receptors and initiate maximal effector modulation, partial, that bind and initiate only a limited effector response, and inverse, that bind to a receptor and decrease the basal level of receptor modulation of effectors.

A mathematical model of receptor-G protein interactions will be defined once the factors involved and their effects have been discussed. Put simply, in the absence of agonist, receptors exist in two affinity states, G protein uncoupled (low affinity) for agonist, and G protein coupled (high affinity) for agonist. The existence of these two different states can be demonstrated using agonist and antagonist radioligand binding studies. Antagonists bind to the two different receptor activity states with equal high affinity, but agonists only bind to the G protein coupled form with high affinity and bind the uncoupled form with low affinity (Kobilka 1992). Two different assays demonstrate this. Firstly, saturation binding analysis of receptor populations using agonists reveals a much lower B_{max} than saturation analysis with an antagonist (Sleight *et al.*, 1996; Chen *et al.*, 1997). Secondly, agonist displacement of antagonist radioligand binding often results in a biphasic competition curve (Waldhoer *et al.*, 1999). The first phase represents the displacement of antagonist following binding of the agonist to the high affinity, or G protein coupled, state of the receptor and the second phase, at higher agonist concentrations, the displacement of antagonist binding following binding of the agonist to the low affinity, or G protein uncoupled, state of the receptor.

The proportion of receptor that is in the high and low affinity states can be influenced by the relative expression levels of the receptor and G protein (Chidiac *et al.*, 1994; MacEwan and Milligan, 1996; Smit *et al.*, 1996). If there is an increase in the levels of G protein expression, there is a resultant increase in the incidence of the G protein coupled, or high affinity, state of the receptor.

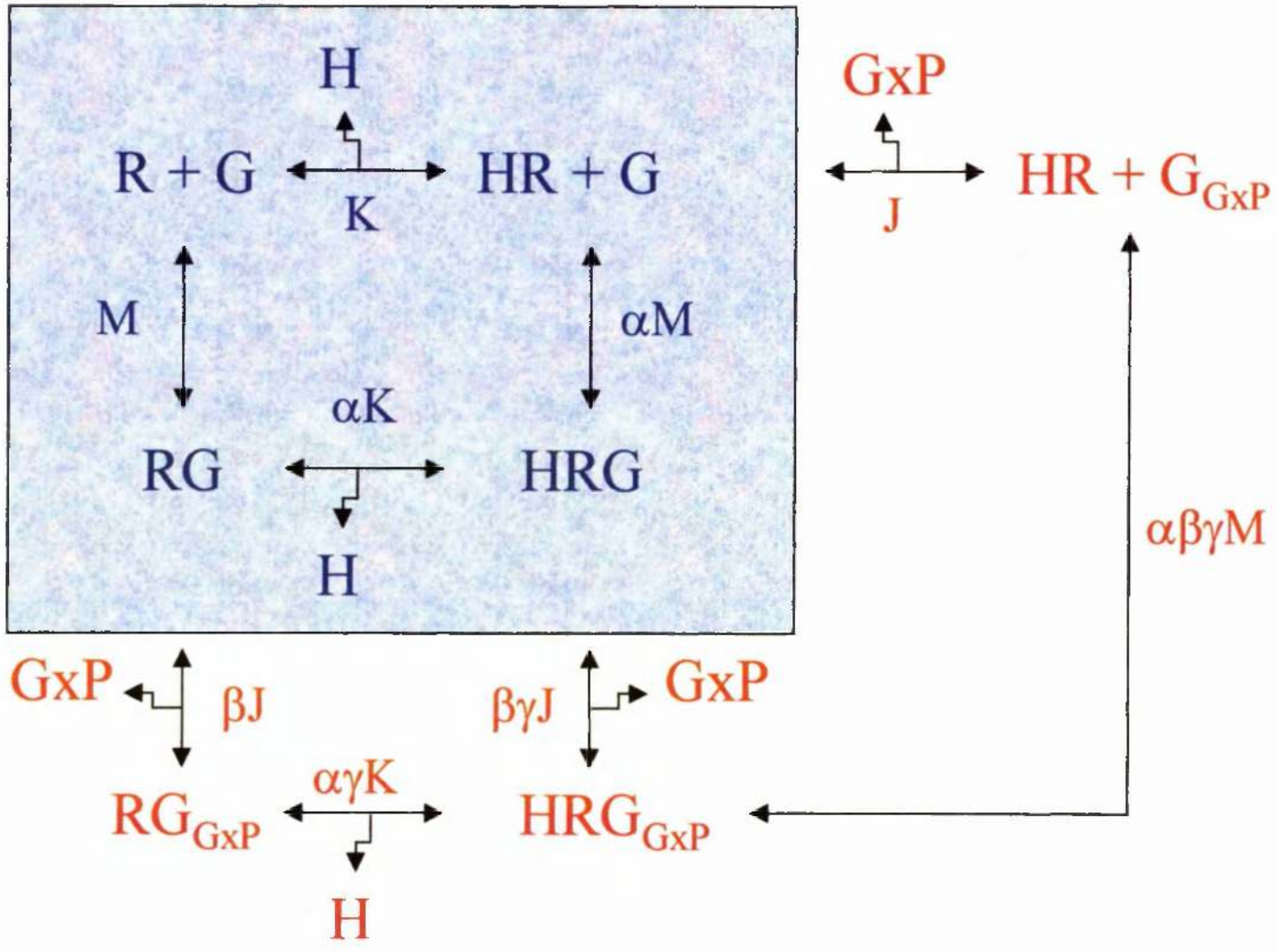
Following agonist activation of a receptor, there is a conformational change in the transmembrane domains and intracellular loops, resulting in an increase in affinity of the receptor for its cognate G protein (Farrens *et al.*, 1996; Bourne *et al.*, 1997; Javitch *et al.*, 1997; Spengler *et al.*, 1993; Pin and Bockaert, 1995; Wess, 1997). This results in an increase in the binding of inactive G protein, i.e. GDP bound, to the receptor, and causes a conformational change in the G protein that decreases the affinity of the G_α subunit for GDP and increases its affinity for GTP. The binding of GTP to the G_α subunit has two effects. Firstly, it results in a decrease in the affinity of the G protein for the receptor and leads to the dissociation of the complex, and secondly it results in a decrease in the affinity of the G_α for the $G_{\beta\gamma}$ subunits and leads to their dissociation into $G_{\alpha\text{-}GTP}$ and $G_{\beta\gamma}$. They subsequently become available to regulate second messenger systems (Hamm, 1998).

This process is short lived due to the intrinsic GTPase activity of the G_α subunit. The GTP is rapidly hydrolysed to GDP (Birnbaumer and Birnbaumer, 1995; Helmreich and Hofmann, 1996; Hamm, 1998), leading to a reversal of the activation process, i.e. an increase in affinity of the G_α for the $G_{\beta\gamma}$ subunits and their reassociation, and also a return to basal affinity of the G protein for the receptor.

The affinity of a receptor for a G protein can be altered by site directed mutagenesis. As mentioned in the previous section, mutations within the DRY motif at the cytosolic end of TM3 often results in a constitutively active mutant receptor (Ren *et al.*, 1993). It is hypothesised that these mutations lead to the exposure of residues in the intracellular loops of the GPCR that are normally only exposed following agonist binding and are involved in the binding of G proteins (Scheer *et al.*, 1996). Their exposure would lead to an increase in the binding of G protein and an increase in the proportion of GPCR in the high affinity, or G protein coupled, state. A second method for increasing the proportion of receptor in the high affinity state is to fuse the N terminal end of a G protein α subunit to the C terminal end of the GPCR (Kellett *et al.*, 1999). This does not alter the affinity of

Figure 1.3 Ternary complex model of receptor-G protein interaction

The ternary complex model is shown in blue, with the effects of GTP and GDP bound states of G protein shown in red. In the figure, the GPCR is represented as R, the G protein as G, the guanyl nucleotide as GxP (either GDP or GTP), and the agonist as H. The symbols K, J and M are affinity constants. Uncoupled receptors have low affinities for both agonist and G protein (K and M respectively). Agonist increases the affinity of receptor for G protein (from M to αM) and vice versa (the allosteric factor α is >1). Guanine nucleotides inhibit the binding of agonists: αγK is lower than αK so γ must be lower than 1. The affinity of GxP for HRG (βγJ) is therefore lower than the affinity of GxP for RG (βJ): agonists inhibit the recognition of nucleotides by receptor-coupled G proteins. Adapted from Waelbroeck (1999).


H
R + G
K
HR + G
M
αM
αK
RG
HRG
H
GxP
J
HR + G_GxP
αβγM
GxP
βJ
βγJ
GxP
αγK
RG_GxP
HRG_GxP
H

the receptor for the G protein or vice versa, but the close proximity of the G protein to the receptor increases the probability of receptor – G protein coupling. Site directed mutagenesis of Cys^{351}, a residue involved in binding to the intracellular loops of a receptor, can result in a G protein with altered affinity for a GPCR (Bahia *et al.*, 1998). The mutation Cys^{351}Ile results in a G protein with an increased affinity for receptor binding, but the mutation Cys^{351}Gly leads to a decrease in affinity. Bahia *et al*, (1998) mutated the residue at this site to each of the 19 other natural amino acids in $G_{i1\alpha}$ and found that the more hydrophobic the residue at position 351, the higher the affinity of the G protein for the receptor.

For the majority of simple binding assays in the presence of agonists and antagonists, the simple ternary complex model of activity would apply (Figure 1.3 blue). This model considers only three G protein species, G protein alone, G protein bound to receptor and G protein bound to ligand activated receptor (De Lean *et al.*, 1980; Costa *et al.*, 1991; Lefkowitz *et al.*, 1993; Weiss *et al.*, 1996a; Weiss *et al.*, 1996b; Colquhoun, 1998). In the absence of GTP, it predicts the existence of 2 agonist binding states, HRG (G protein coupled), and HR (G protein uncoupled), with dissociation constants of $K_H=1/\alpha K$ and $K_L=1/K$ respectively, where K is an affinity constant and α, an allosteric factor. Extension of this model includes the effects of guanyl nucleotides in G protein-receptor interactions (Figure 1.3 red)(Ehlert and Rathbun, 1990; Onaran *et al.*, 1992). As GTP inhibits agonist binding (De Lean *et al.*, 1980; Waelbroeck *et al.*, 1982; Mahle *et al.*, 1992), agonists must decrease the affinity of GTP for RG because allosteric interactions are reciprocal at equilibrium. Waelbroeck (1999) discussed in great detail the effects of guanyl nucleotides, and the limitations of models proposed by Ehlert and Rathbun (1990) and Onaran *et al.*, (1992). Waelbroeck points out although the models are compatible with G protein activation through low-affinity binding sites, they cannot explain catalytic G protein activation because the activated G protein density would have to be lower than the receptor density. The indications are that for each GPCR, the factors that affect the activation state are not only the G protein coupling state of the receptor and the GDP/GTP state of the receptor, but also the ligand that has bound. Each ligand can produce slight differences in the receptor conformation state that will influence affinity for G protein binding.

1.3.3 GPCR-G protein Fusions

The strategy of linking the intracellular C terminal end of a GPCR to the N terminal end of a G protein α subunit has been used to great effect in the study of GPCR-G protein interactions. These constructs ensure a 1: 1 stoichiometry between the receptor and G protein and in the case of pertussis toxin resistant G_α subunit fusions allows for the accurate determination of GTPase activity.

After a slow start where a period of two years passed between the construction of the first GPCR-G protein construct between the β_2-adrenoceptor and $G_{s\alpha}$ (Bertin *et al.*, 1994), this approach has been used to great effect in the study of both a wide variety of GPCRs and G proteins (Milligan 2000; Seifert *et al.*, 1999; Milligan and Rees 1999). The fixed stoichiometry of receptor to G protein allows for the measurement of GTP turnover in a cell expression system (Seifert *et al.*, 1998; Wise *et al.*, 1997; Carr *et al.*, 1998) and the B_{max} from receptor radioligand saturation binding studies can be used to calculate the exact expression of fused G protein α subunits. This information is of particular use when pertussis toxin has been used to ADP ribosylate and uncouple the endogenous pool of inhibitory G proteins, in the presence of a pertussis toxin resistant G protein fusion. Previously the addition of purified proteins in a reconstituted lipid vesicle system was the only method where such accurate measurements could be made (Brandt and Ross 1986; Cerione *et al.*, 1985).

Using both GTPase activity (Seifert *et al.*, 1998, 1999; Wise *et al.*, 1997) and GTPγS binding (Seifert *et al.*, 1998; Wise *et al.*, 1997; Dupuis *et al.*, 1997) the efficacy of partial agonists and inverse agonists have been determined.

The generation of constitutively active receptors has also been used to great advantage in the GTPase assay. These mutants may be generated either by modification of the G protein, where the $Cys^{351}Ile$ mutation in $G_{i1\alpha}$ has been shown to generate constitutive activation (Kellett *et al.*, 1999), or by modification of the receptor (Ren *et al.*, 1993). The expression of these proteins in a mammalian cell line and subsequent assay of GTPase activity is a robust system for screening ligands to measure potential inverse agonist activity (Kellett *et al.*, 1999).

The constructs can also be utilised in traditional radioligand binding studies where the effects of alteration of guanine nucleotide content in a reaction can be

measure in terms of agonist binding (Wenzel-Seifert *et al.*, 1998; Seifert *et al.*, 1999; Waldhoer *et al.*, 1999) providing accurate kinetics for ligand interaction.

1.4 5-HT Receptors

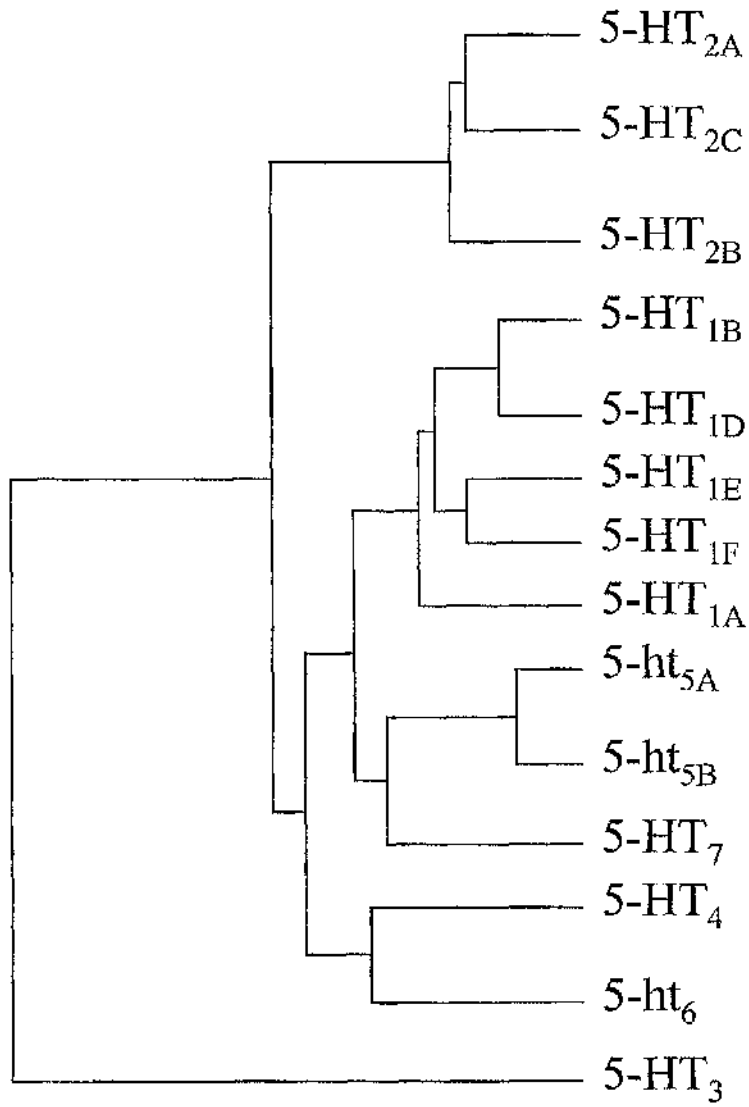
The advent of molecular biological techniques has had a major impact on the discovery and classification of 5-HT receptors. To date, mammalian 5-HT receptors have been divided into seven families, 5-HT_{1-7}, containing 14 receptor subtypes with distinct structural and pharmacological profiles (Hoyer *et al.*, 1994) (Figure 1.4). Of these fourteen members all are predicted to belong to the superfamily of seven transmembrane spanning G protein-coupled receptors with the exception of the 5-HT_3 receptor, which is a ligand gated ion channel. 5-HT has been implicated in the aetiology of many disease states, particularly mental illnesses, such as depression, anxiety, schizophrenia, eating disorders, obsessive compulsive disorder and migraine. Many drugs used to treat these illnesses act by modulating 5-HT signalling. For example, partial 5-HT_{1A} agonists, such as buspirone and gepirone, are effective as anxiolytic (antianxiety) agents (Tunnicliff, 1991; Barret, and Vanover, 1993) and 5-HT_{1A} antagonists enhance the antidepressant effects of selective serotonin uptake inhibitors (S.S.R.I.s) such as fluoxetine (Prozac) (Artigas *et al.*, 1994; Artigas *et al.*, 1996). The development of more selective ligands would hopefully lead to more effective treatments with fewer side effects.

1.4.1 The 5-HT_1 Receptor Family

The 5-HT_1 receptor family consists of five receptor subtypes initially characterised as a [^{3}H]-5-HT binding site in rat cortex with low affinity for spiperone (Peroukta and Snyder, 1979). Subsequent studies of this [^{3}H]-5-HT binding site characterised the 5-HT_{1A} and 5-HT_{1B} receptors (Pedigo *et al.*, 1981; Middlemiss and Fozard, 1983), the 5-HT_{1C} receptor, that has now been reclassified as the 5-HT_{2C} receptor (Pazos *et al.*, 1984), the 5-HT_{1D} receptor (now recognised as a species variant of the 5-HT_{1B} and the closely related 5-HT_{1D} receptors)(Hoyer *et al.*, 1985 a,b; Heuring and Peroukta, 1987), 5-HT_{1E} receptor (Leonhardt *et al.*, 1989), and 5-HT_{1F} receptor (Amlaiky *et al.*, 1992; Adham *et al.*, 1993a,b). These receptors have high sequence homology (>40%) and all couple negatively to adenylyl cyclase *via* G proteins.

Figure 1.4 **Dendrogram illustration of the evolutionary relationship between various 5-HT receptor protein sequences**

The relationship is as defined in Barnes and Sharp (1999), where the multiple sequence alignments were created using a simplification of the progressive alignment method of Feng and Doolittle (1987).


5-HT2A
5-HT2C
5-HT2B
5-HT1B
5-HT1D
5-HT1E
5-HT1F
5-HT1A
5-ht5A
5-ht5B
5-HT7
5-HT4
5-ht6
5-HT3

1.4.1.1 The 5-HT_{1A} Receptor

The rapid characterisation of this receptor following its identification (Pedigo *et al.*, 1981; Middlemiss and Fozard, 1983) was due in part to the early development of the 5-HT_{1A} selective agonist 8-OH-DPAT (Hjorth *et al.*, 1982). This ligand is now known to bind at relatively high concentrations to the 5-HT_7 receptor (Plassat *et al.*, 1993; Tsou *et al.*, 1994; Nelson *et al.*, 1995). The 5-HT_{1A} receptor was the first 5-HT receptor to be fully sequenced and was identified as a sequence homologous to the β_2-adrenoceptor (Kobilka *et al.*, 1987; Fargin *et al.*, 1988; Albert *et al.*, 1990). It has been established that Asn^{385} in TM7 confers the high affinity of the receptor for some β-adrenergic ligands (Guan *et al.*, 1992). The original sequence of the human 5-HT_{1A} receptor (Kobilka *et al.*, 1987) contained a sequencing error near the junction of the second intracellular loop and TM4, and was modified (Chanda *et al.*, 1993). The encoded human protein has 422 amino acids with a relative molecular weight of approximately 46,000. The human receptor is located on chromosome 5 (5q11.2-q13) and the gene is intronless with a predicted typical GPCR structure.

[^{3}H]-8-OH-DPAT was synthesised in 1983 (Gozlan *et al.*, 1983) and allowed the 5-HT_{1A} receptor distribution to be mapped and binding pharmacologically characterised. Autoradiography using a range of radioligands, [^{3}H]-5-HT, [^{3}H]-8-OH-DPAT, [^{3}H]-WAY100635, [^{125}I]-MPPI has mapped brain 5-HT_{1A} distribution extensively (Pazos and Palacios, 1985; Hoyer *et al.*, 1986; Khawaja 1995; Kung *et al.*, 1995). More recently in living human brain, PET studies using [^{11}C]-WAY100635 have been used to map 5-HT_{1A} receptors (Pike *et al.*, 1995). These studies have shown high density of 5-HT_{1A} sites in the hippocampus, lateral septum, cortical areas, and in both dorsal and median raphe nuclei and low levels of 5-HT_{1A} binding sites in the basal ganglia and cerebellum. This pattern of radioligand binding is almost identical to the distribution of 5-HT_{1A} mRNA (Chalmers and Watson 1991; Miquel *et al.*, 1991; Pompeiano *et al.*, 1993; Burnet *et al.*, 1995). The 5-HT_{1A} receptor is located both pre- (in the mesencephalic and medullary raphe nuclei) and post-synaptically (in forebrain regions) (Miquel *et al.*, 1991, 1992; Radja *et al.*, 1991).

The 5-HT_{1A} receptor is clearly set apart from the 5-HT_1 and other 5-HT receptors by its selectivity for a number of ligands (Hoyer *et al.*, 1994). The receptor selectively binds the agonists 8-OH-DPAT, gespirone and dipropyl-5-CT and a

number of antagonists, (S)-UH-301, WAY100135, WAY100635 (most potent) (Hillver *et al.*, 1990; Bjork *et al.*, 1991; Fletcher *et al.*, 1993a,b and 1996) and recently NAD-299 which appears to be the most selective (Johansson *et al.*, 1997). WAY100135 has been shown to have some partial agonist activity in some systems (Davidson *et al.*, 1997; Schoeffter *et al.*, 1997). Another highly efficacious agonist at the 5-HT_{1A} receptor, S 14506 (Colpaert *et al.*, 1992), has some interesting properties. It is structurally related to the inverse agonist spiperone (Barr and Manning, 1997; Newman-Tancredi *et al.*, 1997a,b; Kellett *et al.*, 1999; Mcloughlin and Strange, 2000) and behaves as one of the most potent agonists both *in vitro* and *in vivo*, but has binding characteristics more akin to an antagonist (Milligan *et al.*, 2001). It was proposed that S 14506 interacted with the 5-HT_{1A} receptor not only at the 5-HT binding site, but also at the DRY motif that is involved in G protein coupling.

The 5-HT_{1A} receptor is negatively coupled to adenylyl cyclase through activation of inhibitory G proteins as demonstrated in both rat and guinea pig tissue and cell lines stably expressing the cloned receptor (Boess and Martin 1994; Saudou and Hen, 1994; Albert *et al.*, 1996). Despite its high expression in dorsal raphe, the 5-HT_{1A} receptor does not appear to inhibit cAMP production there (Clarke *et al.*, 1996). There have also been reports of positive coupling to adenylyl cyclase of the 5-HT_{1A} receptor (Shenker *et al.*, 1983; Markstein *et al.*, 1986), but given the similarities between the 5-HT_{1A} and 5-HT_7 receptors, these results may simply have been misinterpreted.

The mechanism of 5-HT_{1A} mediated inhibition of adenylyl cyclase is still unclear. The adenylyl cyclase family consists of at least nine members that are regulated by a number of different mechanisms (Hurley, 1999; Mons *et al.*, 1998; Taussig and Zimmerman, 1998), reflected in the ability of $G_{i\alpha}$ proteins to bind and inhibit types 5 and 6 adenylyl cyclase, $G_{\beta\gamma}$ subunits stimulating type 2 adenylyl cyclase when activated $G_{s\alpha}$ is present, elevations in intracellular Ca^{2+} inhibiting types 5 and 6 adenylyl cyclase, Ca^{2+}/calmodulin stimulating types 1, 3 and 8 adenylyl cyclases, whereas Ca^{2+}/calmodulin-dependent protein kinase II inhibits type 3 adenylyl cyclase, and finally, protein kinase C stimulating types 2 and 7.

The activation of PI-PLC by 5-HT_{1A} receptors was first demonstrated in HeLa cells (Fargin *et al.*, 1989) and was shown to be as effective as coupling *via* endogenous H_1 receptors (Raymond *et al.*, 1990). Activation was also shown in

human Jurkat cells (Aune *et al.*, 1993) and the mechanism was demonstrated in Lkt$^-$ fibroblasts to be by 5-HT increased phosphoinositide and intracellular levels of Ca^{2+} (Liu and Albert 1991) and that in both Lkt$^-$ and BALB/c-3T3 cells, the increase in intracellular Ca^{2+} was from release from intracellular stores (Abdel-Baset 1992).

The 5-HT_{1A} receptor is coupled to protein kinase C activation (Middleton *et al.*, 1990; Raymond *et al.*, 1989) in HeLa cells where it is dependent on PLC activation (Fargin *et al.*, 1989). The effect is probably mediated via $G_{\beta\gamma}$ and is dependent on the expression of $G_{\beta\gamma}$ regulatable PKC (Raymond *et al.*, 1999). 5-HT_{1A} mediated activation of PLA_2 has been demonstrated in HeLa cells (Harrington *et al.*, 1994), as has augmentation of Ca^{2+} induced arachidonic acid metabolism in CHO cells (Raymond *et al.*, 1992).

The 5-HT_{1A} receptor has demonstrated coupling to a number of ion channels, including the G protein coupled inwardly rectifying K^+ channel (Andrade and Nicoll 1986; Colino and Halliwell 1987; Zgombick *et al.*, 1987), via interaction with $G_{\beta\gamma}$ subunits released on receptor activation (Doupnik *et al.*, 1996). In native systems, inactivation of 5-HT_{1A} stimulated GIRK channel opening is 20-40 times faster than in systems where the cloned receptor and channel have been co-expressed (Andrade and Nicoll 1986; Colino and Halliwell 1987; Zgombick *et al.*, 1987; Dascal *et al.*, 1993; Karschin *et al.*, 1991). It was suggested that additional components, in this case, regulators of G protein signalling (RGS) proteins, may be involved in the native pathway (Doupnik *et al.*, 1997). It was demonstrated that kinetics similar to those of the native systems could be restored following co-expression of the 5-HT_{1A} receptor, GIRK1, and one of RGS1, RGS3, and RGS4 proteins, but that co-expression with RGS2 protein did not restore the rapid native kinetics (Doupnik *et al.*, 1997).

Regulation of a number of other channels by 5-HT_{1A} receptor activation has been demonstrated. The 5-HT_{1A} receptor can stimulate an oscillatory Ca^{2+} activated Cl^- current in *Xenopus* oocytes (Ni *et al.*, 1997) as well as augment the activation of CFTR Cl^- channels induced by β_2-adrenergic receptors (Uezono *et al.*, 1993). Inhibition of Bay K8644-mediated Ca^{2+} influx in GH_4C_1 cells by rat 5-HT_{1A} receptor activation has also been demonstrated and this requires co-expression of $G_{o\alpha}$ (Liu and Albert, 1991; Liu *et al.*, 1994).

The coupling of the 5-HT_{1A} receptor to the different types of inhibitory G proteins has been examined in many systems. The 5-HT_{1A} receptor has been co-

expressed with mammalian G proteins in *Spodoptera frugiperda* (Sf9) cells (Barr *et al.*, 1997; Butkerait *et al.*, 1995), where co-expression of $G_{i\alpha}$ proteins and various combinations of β_1 and γ subunits increased the affinity for agonists. When the 5-HT_{1A} receptor was co-expressed with β_1 and γ_2, relatively equivalent coupling to α_{i1}, α_{i2}, α_{i3}, α_o and α_z was seen with no coupling to α_s and α_q (Barr *et al.*, 1997; Butkerait *et al.*, 1995). Co-expression of the 5-HT_{1A} with β_1 and α_{i1} and various γ subunits revealed an order of preference for coupling to $\gamma_2 \cong \gamma_3 \cong \gamma_5 > \gamma_1$ (Barr *et al.*, 1997; Butkerait *et al.*, 1995).

Expression of the 5-HT_{1A} receptor in *E.coli* showed a rank order of affinity for reconstituted purified mammalian G protein α subunits of $G_{i3\alpha} > G_{i2\alpha} > G_{i1\alpha} >> G_{o\alpha} >> G_{s\alpha}$ (Bertin *et al.*, 1992). In HeLa and CHO cells, agonist induced coupling of the 5-HT_{1A} receptor and G proteins revealed an apparent rank order of $G_{i3\alpha} > G_{i2\alpha} \cong G_{i1\alpha} \cong G_{o\alpha} > G_{z\alpha} >> G_{s\alpha}$ (Garnovskaya *et al.*, 1997; Raymond *et al.*, 1993).

Linkage of 5-HT_{1A} signalling through specific G_α proteins to a number of second messenger systems has been demonstrated. The 5-HT_{1A} receptor in HeLa cells was shown to inhibit adenylate cyclase and activate PI-PLC via $G_{i3\alpha}$ (Fargin *et al.*, 1991) and also in CHO and HeLa cells, 5-HT_{1A} mediated inhibition of adenylyl cyclase was demonstrated by $G_{i1\alpha}$, $G_{i2\alpha}$ and $G_{i3\alpha}$ (Raymond *et al.*, 1993), findings that are unsurprising as inhibition of cAMP accumulation in mammalian cells following 5-HT_{1A} receptor activation was demonstrated via $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$ and $G_{z\alpha}$ (Wong *et al.*, 1992). These, among other studies, have suggested different effectiveness for various $G_{i\alpha}$ proteins in either CHO or HeLa cells (Raymond *et al.*, 1993; Wong *et al.*, 1992; Gettys *et al.*, 1994), but these results may have been affected by differences in the endogenous pools of G protein. In HeLa cells, $G_{i3\alpha}$ is more highly expressed than $G_{i2\alpha}$, and in CHO cells, $G_{i2\alpha}$ is expressed at levels approximately nine fold of those of $G_{i3\alpha}$ (Raymond *et al.*, 1993). A number of studies suggest that the 5-HT_{1A} receptor is linked to inhibition of adenylate cyclase and activation of PLC through $G_{i1\alpha}$ or $G_{i3\alpha}$ and to the inhibition of Ca^{2+} channels via $G_{o\alpha}$ (Liu *et al.*, 1994; Albert *et al.*, 1996; Langlois *et al.*, 1996).

The 5-HT_{1A} receptor has been implicated in growth stimulation (Ishizuka *et al.*, 1992). It has been reported to induce the secretion of a growth factor (protein S-100) from primary astrocyte cultures (Azmitia *et al.*, 1996) and increase markers of

growth in neuronal cultures (Riad *et al.*, 1994), findings that raise the possibility of the 5-HT_{1A} receptor having a neurotrophic role in developing and adult brain (Riad *et al.*, 1994; Azmitia *et al.*, 1996; Yan *et al.*, 1997). The 5-HT_{1A} receptor has also been linked to the activation of ERK (extracellular signal-regulated kinase) and NF-κB (nuclear factor-κB) (Cowen *et al.*, 1996, 1997; Luttrell *et al.*, 1997; Marshall 1995; Garnovskaya *et al.*, 1996, 1998).

A number of important structural features have been identified in the 5-HT_{1A} receptor. The high affinity of the 5-HT_{1A} receptor for β-adrenoceptor blockers such as pindolol (Guan *et al.*, 1992) has been attributed to a single amino acid residue, Asn^{386} in TM7, which is uniquely conserved in the 5-HT_{1A} receptor and all β-adrenoceptors. The mutation $Asn^{386}Val$ decreased the affinity for binding of pindolol and similar ligands but did not effect the binding of 5-HT_{1A} receptor ligands. Other mutations in the TM domains have resulted in decreased affinity for 5-HT (Ho *et al.*, 1992). The mutations $Asp^{82}Asn$, $Asp^{116}Asn$ and $Ser^{199}Ala$ decreased the affinity for 5-HT binding by 60-100 fold but did not effect pindolol binding. Binding of 5-HT to the $Thr^{200}Ala$ mutant was undetectable.

The use of synthetic peptides from the second and third intracellular loops of the 5-HT_{1A} receptor suggested sites of G protein interaction (Varrault *et al.*, 1994). A peptide consisting of the entire second intracellular loop (Asp^{133}-Arg^{153}) and a region of the third intracellular loop (Ala^{331}-Leu^{347}), but not the (Ala^{336}-Val^{344}) peptide, inhibited forskolin stimulated cAMP production in membranes from NIH-3T3 cells, Sf9 cells and rat hippocampus and increased GTPγS binding to purified bovine $G_{i/o\alpha}$ proteins, indicating the importance of these site in G protein coupling. A role for Thr^{149} in the second intracellular loop has also been demonstrated in mediating $G_{\beta\gamma}$ specific responses (Albert *et al.*, 1998).

A number of serine and threonine sites in the second and third intracellular loops of the 5-HT_{1A} receptor have been identified as putative PKC phosphorylation sites and play a role in signalling specificity and receptor desensitisation (Lembo *et al.*, 1995, 1997). Single mutants of the 5-HT_{1A} receptor, $Thr^{229}Ala$, $Ser^{253}Gly$ and $Thr^{343}Ala$ had no effect on PKC mediated desensitisation of Ca^{2+} mobilisation in Ltk^- fibroblasts, but the double ($Thr^{229}Ala$ and $Ser^{253}Gly$) and triple mutant ($Thr^{229}Ala$, $Ser^{253}Gly$ and $Thr^{343}Ala$) were progressively more resistant to PKC mediated desensitisation. Lembo *et al.* (1997) also found that the $Thr^{149}Ala$ mutant inhibited

elevation of Ca^{2+} in Ltk$^-$ cells and was unable to inhibit opening of BayK8644 sensitive Ca^{2+} channels in GH_4C_1 cells while only partially uncoupling the 5-HT_{1A} receptor from inhibition of cAMP production. This work suggests that residues Thr^{229}, Ser^{253} and Thr^{343} in the third intracellular loop mediate the desensitisation of the rat 5-HT_{1A} receptor, that Thr^{229} is important for coupling to inhibition of adenylyl cyclase and elevations in intracellular Ca^{2+} and that Thr^{149} in the second intracellular loop has a role in elevation of intracellular Ca^{2+} and inhibition of adenylyl cyclase.

A technique that has been used to investigate the function of a specific gene *in vivo* is the generation of "knockout" animal models. Two groups have used this technique to generate mouse lines that lack 5-HT_{1A} receptors and have assessed the effects this had in terms of behavioural models of anxiety and stress, disease states in which particularly the presynaptic 5-HT_{1A} receptor has been implicated (Ramboz *et al.*, 1998; Heisler *et al.*, 1998). Neither group found any difference in the serotonin content of knockout versus WT mice or in the amount of serotonin released following electrical stimulation of slices from the hippocampus or mesencephalic regions. In WT mice, 8-OH-DPAT produced a 30-40% reduction in this release but had no effect in the knockout mice (Ramboz *et al.*, 1998). These findings could either indicate that 5-HT_{1A} receptor does not play a significant role in the regulation of serotonin release or that in its absence another receptor is fulfilling the role.

A possible candidate for 5-HT_{1A} functional substitution is the 5-HT_{1B} receptor. As with the 5-HT_{1A} receptor it is negatively coupled to adenylyl cyclase and is also expressed presynaptically at the axon terminal (Martin *et al.*, 1998; Hammon 1997). Ramboz *et al.* (1998) suggested that the 5-HT_{1B} receptor may be upregulated in 5-HT_{1A} knockouts and demonstrated that both in WT and knockouts, 5-HT_{1B} agonists were able to attenuate electrically evoked serotonin release.

Both groups examined the behaviour of the mice in terms of anxiety, with an increased level of anxiety shown as a decreased level of exploratory behaviour and vice versa. In both studies, the 5-HT_{1A} knockout mice showed a decreased level of exploration when compared to WT mice with the Ramboz study indicating that only male knockout mice showed a significantly decreased level of exploration. Levels of depression were also measured using two similar models, the forced swim test and the tail suspension test, both of which record increased immobility as an indicator of increased depression. Again the results from the two groups were the same, with the

knockout mice recording shorter periods of immobility compared to WT mice indicating that a lack of functional 5-HT_{1A} receptor favours a less depressed state.

These models are helpful in furthering understanding of 5-HT_{1A} receptor function but more stringent studies were suggested by both groups. Heisler *et al.* (1998) suggested both microdialysis and electrophysiological studies, whereas Ramboz *et al.* (1998) suggested using a tissue specific knockout approach (Kuhn *et al.*, 1995).

1.4.1.2 The 5-HT_{1B} Receptor

The 5-HT_{1B} serotonin receptor was identified at the same time as the 5-HT_{1A} receptor as a [^{3}H]-5-HT binding site in rodent brain with low affinity for spiperone (Pedigo *et al.*, 1981). However, its low affinity for 8-OH-DPAT, the selective 5-HT_{1A} receptor agonist, set it apart pharmacologically (Middlemiss and Fozard 1983). The gene encoding the human 5-HT_{1B} receptor is located on chromosome 6 (6q13) (Saudou and Hen, 1994) and the receptor is expressed highly in the rat basal ganglia and other regions (Pazos *et al.*, 1985; Verge *et al.*, 1986; Bruinvels *et al.*, 1993). There are a large number of ligands available to study the 5-HT_{1B} receptor, but few of these show any selectivity (Hoyer *et al.*, 1994). The most potent agonists include 5-CT and RU 24969, with methiothepin being a potent antagonist. However, these ligands have affinity for other 5-HT receptors, particularly the 5-HT_{1A} receptor. Some selectivity has been provided by the antagonist GR 127935 (Skingle *et al.*, 1995) which has shown high selectivity for the 5-$HT_{1B/1D}$ over other 5-HT receptors and the antagonists SB-224289 and SB-216641 which show selectivity 5-HT_{1B} over 5-HT_{1D} (Price *et al.*, 1997; Roberts *et al.*, 1997).

The 5-HT_{1B} receptor couples negatively to forskolin stimulated adenylyl cyclase (Adham *et al.*, 1992; Levy *et al.*, 1992a; Weinshank *et al.*, 1992). A number of compounds, including methiothepine, ketanserin and SB-224289 have shown inverse agonist activity at the 5-HT_{1B} receptor using [^{35}S]-GTPγS binding (Pauwels *et al.*, 1997; Roberts *et al.*, 1997). There is also evidence supporting the role of 5-HT_{1B} receptors as autoreceptors on 5-HT nerve terminals (Middlemiss and Huston, 1990; Buhlen *et al.*, 1996).

1.4.1.3 The 5-HT_{1D} Receptor

The classification of the 5-HT_{1D} receptor was a long process that involved confusion with the 5-HT_{1B} receptor and definition as 5-$HT_{1D\alpha/\beta}$ isoforms (Hamblin and Metcalf 1991; Levy *et al.*, 1992a,b; Weinshank *et al.*, 1992). However, the 5-$HT_{1D\beta}$ was redefined as a species homologue of the 5-HT_{1B} receptor and with the discovery of a rat gene homologous to the 5-$HT_{1D\alpha}$ receptor with a 5-HT_{1D} binding site profile (Hamblin *et al.*, 1992), the 5-$HT_{1D\alpha}$ receptor was renamed the 5-HT_{1D} receptor (Hartig *et al.*, 1996). Due to a lack of 5-HT_{1D} selective radioligands the distribution of the receptor is poorly defined. *In situ* hybridisation studies have detected 5-HT_{1D} mRNA in various rat regions including caudate putamen, olfactory cortex and dorsal raphe nucleus (Hamblin *et al.*, 1992; Bruinvels *et al.*, 1994a,b). Available data indicate on the whole that the 5-HT_{1D} receptor is present mainly on axon terminals of both 5-HT and non 5-HT neurones.

The 5-HT_{1D} receptor has a binding profile that is almost indistinguishable from the 5-HT_{1B} receptor (Weinshank *et al.*, 1992; Boess and Martin, 1994). Only a few compounds show any selectivity for the 5-HT_{1D} receptor over the 5-HT_{1B} receptor, ketanserin and ritanserin being only 15-30 fold selective (Kaumann *et al.*, 1994; Pauwels *et al.*, 1996) and BRL-15572 only 60 fold selective 5-HT_{1D} over 5-HT_{1B} (Price *et al.*, 1997). The 5-HT_{1D} couples negatively to adenylyl cyclase activation (Hamblin and Metcalf 1991; Weinshank *et al.*, 1992).

1.4.1.4 The 5-HT_{1E} Receptor

The 5-HT_{1E} receptor was identified as a novel 5-HT receptor in radioligand bindings using selective ligands to block the 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C} (now the 5-HT_{2C}) receptors and distinguishable from the 5-HT_{1D} receptor by having a low affinity for 5-CT (Waeber *et al.*, 1988; Leonhardt *et al.*, 1989). It was subsequently isolated (McAllister *et al.*, 1992; Zgombick *et al.*, 1992). The 5-HT_{1E} receptor gene is intronless, encodes a protein of 365 amino acids (McAllister *et al.*, 1992; Zgombick *et al.*, 1992; Gudermann *et al.*, 1993), and is located on chromosome 6q14-q15 (Levy *et al.*, 1992b). There are currently no selective ligands for the 5-HT_{1E} receptor, but it is characterised by its high affinity for 5-HT and lower affinity for 5-CT, as well as low affinity for β-adrenergic ligands (Adham *et al.*, 1994). The receptor has shown modest inhibition of adenylyl cyclase activity, with methiothepin

acting as a weak antagonist and 5-CT as a weak agonist (McAllister *et al.*, 1992; Zgombick *et al.*, 1992; Levy *et al.*, 1992a).

1.4.1.5 The 5-HT_{1F} Receptor

The 5-HT_{1F} receptor was initially detected in mouse (Amlaiky *et al.*, 1992) and later in human (Adham *et al.*, 1993b) having initially been described as a 5-$HT_{1E\beta}$ receptor (Amlaiky *et al.*, 1992). The receptor shows a similar low affinity to the 5-HT_{1E} receptor for 5-CT, but markedly different mRNA distribution in the brain. As with the other members of the 5-HT_1 family it is intronless with the gene located on chromosome 3q11 (Saudou and Hen 1994). As with the 5-HT_{1E}, the 5-HT_{1F} receptor has a high affinity for 5-HT and a low affinity for 5-CT but shows a high affinity for sumatriptan whereas the 5-HT_{1E} receptor does not (Amlaiky *et al.*, 1992; Adham *et al.*, 1993a,b; Lovenberg *et al.*, 1993a,b). There are two selective 5-HT_{1F} agonists available, LY 344864 and LY 334370 (Overshiner *et al.*, 1996; Johnson *et al.*, 1997; Phebus *et al.*, 1997). The receptor is negatively coupled to forskolin stimulated adenylyl cyclase (Amlaiky *et al.*, 1992; Adham *et al.*, 1993a,b; Lovenberg *et al.*, 1993a,b) with 5-HT acting as a potent agonist and methiothepine as a weak antagonist. The selective 5-HT_{1F} receptor ligands, LY 344864 and LY 334370 are potent agonists when inhibiting cAMP accumulation (Johnson *et al.*, 1997; Phebus *et al.*, 1997).

1.4.2 The 5-HT_2 Receptor Family

There are currently three members of the 5-HT_2 receptor family, the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. The 5-HT_{2A} receptor was originally called the 5-HT D receptor (Gaddum and Picarelli 1957) and the 5-HT_{2C} replaced the 5-HT_{1C} receptor (Humphrey *et al.*, 1993; Hoyer *et al.*, 1994). The amino acid sequences of the 5-HT_2 receptors are highly homologous within the 7TM domains but are distinct from the other 5-HT receptors (Baxter *et al.*, 1995). All 5-HT_2 receptor genes have two (2A and 2B), or three introns (2C) in the coding sequence (Yu *et al.*, 1991; Chen *et al.*, 1992; Stam *et al.*, 1992) and all couple positively to PLC and mobilise intracellular calcium.

1.4.2.1 The 5-HT_{2A} Receptor

5-HT_{2A} binding sites have a high affinity for spiperone, but relatively low affinity for 5-HT (μM) (Leysen *et al.*, 1978; Peroukta and Snyder, 1979). The human 5-HT_{2A} receptor is located on chromosome 13q14-q21 and has a high sequence homology with the 5-HT_{2C} receptor but lower with the 5-HT_{2B} receptor (Pritchett *et al.*, 1988; Julius *et al.*, 1990). The receptor has five possible glycosylation sites, approximately eleven possible phosphorylation sites and a single site for palmitoylation (Saltzman *et al.*, 1991). The antagonist MDL 100907 is selective for and potent at the 5-HT_{2A} receptor, with lower affinity for the 5-HT_{2C} and other receptors (Sorenson *et al.*, 1993; Kehne *et al.*, 1996). The 5-HT_{2A} receptor couples positively to PLC leading to increased accumulation of inositol phosphates and intracellular Ca^{2+} (Boess and Martin 1994). It has also been linked to altered expression of a number of genes including the brain-derived neurotrophic factor (BDNF) (Vaidya *et al.*, 1997).

1.4.2.2 The 5-HT_{2B} Receptor

This receptor was originally classed as 5-HT_1 like (Bradley *et al.*, 1986) and was responsible for the contraction of the rat stomach fundus (Vane 1959). Schmuck *et al.* (1994) isolated what was originally termed the 5-HT_{2F} receptor (Kursar *et al.*, 1992) but this was later reclassified as the 5-HT_{2B} receptor (Humphreys *et al.*, 1993). It has 481 amino acids, two introns that are present at positions corresponding to those of the 5-HT_{2A} and 5-HT_{2C} receptor genes and is located on chromosome 2q36.3-2q37.1 (Foguet *et al.*, 1992). The novel antagonist SB 204741 is 20-60 fold selective for the 5-HT_{2B} receptor over the 5-HT_{2A}, 5-HT_{2C} and other receptors (Baxter *et al.*, 1995; Bonhaus *et al.*, 1995; Baxter 1996). The cloned 5-HT_{2B} receptor stimulates phosphoinositol hydrolysis (Wainscott *et al.*, 1993; Kursar *et al.*, 1994; Schmock *et al.*, 1994). The receptor may also have a role in neural development indicated by severe neural abnormalities in knockout 5-HT_{2B} mice (Choi *et al.*, 1997; Nebigil *et al.*, 1998).

1.4.2.3 The 5-HT_{2C} Receptor

This receptor was originally termed the 5-HT_{1C} due to its high affinity for [^{3}H]-5-HT (Razos *et al.*, 1984) but once it was cloned and further characterised it

was reclassified as the 5-HT_{2C} receptor (Humphreys *et al.*, 1993). A splice variant of the 5-HT_{2C} receptor has been found in rat, mouse and human (Canton *et al.*, 1996), but a functional role is unclear as it lacks a 5-HT binding site. The receptor is X-linked (human chromosome Xq24) and has three introns and may produce a GPCR with 8TM domains, however this has yet to be demonstrated (Yu *et al.*, 1991). The antagonists SB 242084 and RS-102221 are at least 10-100 fold selective for the 5-HT_{2C} receptor over the 5-HT_{2A}, 5-HT_{2B} and other receptors (Bonhaus *et al.*, 1997; Kennett *et al.*, 1997a,b). The 5-HT_{2C} receptor increases PLC activity in the choroid plexus and stably transfected cell lines *via* a G protein coupled mechanism (Boess and Martin 1994).

1.4.3 The 5-HT_3 Receptor

The 5-HT_3 receptor is the exception of the 5-HT receptor family as it is a ligand gated ion channel (Derkach *et al.*, 1989; Maricq *et al.*, 1991). It is a multiple subunit channel with genes encoding the 5-HT_{3A} subunit (Maricq *et al.*, 1991) and the 5-HT_{3B} subunit (Davies *et al.*, 1999) having been identified. A spliced variant of the 3A subunit has been identified (5-HT_{3AS} or short) (Hope *et al.*, 1993) mRNA for which is 4-6 times more abundant in mouse neuronal tissue (Werner *et al.*, 1994). The ion channel is cation selective ($K^+ \cong Na^+$) and is prone to rapid desensitisation (Jackson and Yakel 1995). The receptor has a selective antagonist, MDL 72222, which shows varying interspecies affinity (Kilpatrick and Tyers 1992) and is associated with fast synaptic transmission in the brain (Sugita *et al.*, 1992).

1.4.4 The 5-HT_4 Receptor

The 5-HT_4 receptor was identified in cultured mouse colliculi neurones and guinea pig brain (Dumuis *et al.*, 1988; Bockaert *et al.*, 1990). There are four isoforms of the receptor, 5-HT_{4S} (short) and 5-HT_{4L} (long) (Gerald *et al.*, 1995), renamed 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ respectively by the recommendations of IUPHAR (Hoyer and Martin 1997), and 5-$HT_{4(c)}$ and 5-$HT_{4(d)}$ (Blondel *et al.*, 1998: Bockaert *et al.*, 1998). A number of highly selective antagonists are available for the 5-HT_4 receptors, with GR 113808 and SB 204070 among them. The receptors all couple positively to adenylyl cyclase (Gerald *et al.*, 1995; Claysen *et al.*, 1996), with as yet no distinguishable differences between the various isoforms.

1.4.5 The 5-ht$_5$ Receptor Family

There are two 5-ht$_5$ receptors, the 5-ht$_{5a}$ and 5-ht$_{5b}$, both of which were identified from a mouse brain cDNA library (Plassat *et al.*, 1992a,b; Hen 1992). The human 5-ht$_{5a}$ receptor has also been isolated (Rees *et al.*, 1994). In accordance with IUPHAR nomenclature, lower case lettering is used as functional activity has yet to be demonstrated for the 5-$_{ht5}$ receptors. Both genes contain an intron in a position corresponding to the middle of the third intracellular loop (Matthes *et al.*, 1993). Both receptors have relatively high affinity for 5-CT, LSD, methiothepin and sumatriptan (Erlander *et al.*, 1993; Matthes *et al.*, 1993). Using multiple expression systems, neither the 5-ht$_{5a}$ or 5-ht$_{5b}$ receptors have demonstrated coupling to either adenylyl cyclase or inositol phosphates (Plassat *et al.*, 1992a,b; Erlander *et al.*, 1993; Metthes *et al.*, 1993; Wisden *et al.*, 1993), although in HEK293 cells at levels of expression exceeding 25pmol/mg protein there was a report of inhibition of forskolin stimulated adenylyl cyclase activity (Fracken *et al.*, 1998).

1.4.5.1 The 5-ht$_{5a}$ Receptor

The receptor is predicted to have 357 amino acids (Plassat *et al.*, 1992a,b; Rees *et al.*, 1994; Erlander *et al.*, 1993) and contains consensus sequences for both N-linked glycosylation in the N terminal tail and PKC sensitive phosphorylation sites in the intracellular loops. The 5-ht$_{5a}$ gene is located on human chromosome 7q36 (Matthes et al., 1993).

1.4.5.2 The 5-ht$_{5b}$ Receptor

The 5-ht$_{5b}$ receptor is predicted to contain 370-371 amino acids in mouse or rat (Erlander *et al.*, 1993; Mathes *et al.*, 1993; Wisden *et al.*, 1993) and has consensus sequences for N-linked glycosylation in the N terminus and for PKC sensitive phosphorylation on the intracellular domains. The 5-ht$_{5b}$ receptor gene is located on human chromosome 2q11-13 (Metthes et al., 1993).

1.4.6 The 5-ht$_6$ Receptor

Following some differences in original sequences of the 5-ht$_6$ receptor cDNAs, the reports were reconciled (Kohen *et al.*, 1996; Boess *et al.*, 1997). The human 5-ht$_6$ receptor comprises 440 amino acids and is predicted to be a member of

the GPCR superfamily (Kohen *et al.*, 1996). It has a consensus sequence for N-linked glycosylation on the N terminus and a number of predicted phosphorylation sites on the third intracellular loop and C terminal tail (Kohen *et al.*, 1996). It undergoes agonist-induced desensitisation following phosphorylation catalysed by cAMP dependent protein kinase (Sleight *et al.*, 1997) with studies indicating that the 5-ht_6 receptor is positively coupled to adenylyl cyclase (Sebben *et al.*, 1994; Schoeffter and Waeber 1994). The human 5-ht_6 receptor gene is located on chromosome 1p35-36. Two 5-ht_6 selective antagonists are available, Ro 04-6790 and Ro 63-0563 (Sleight *et al.*, 1998).

1.4.7 The 5-HT_7 Receptor

Despite it being the most recently cloned 5-HT receptor, functional responses corresponding to the 5-HT_7 receptor were recorded prior to its cloning (Eglen *et al.*, 1997). It has been identified in a large number of species including mouse, rat, guinea pig and human (Bard *et al.*, 1993; Lovenberg *et al.*, 1993a,b; Meyerhof *et al.*, 1993; Plasat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Tsou *et al.*, 1994; Nelson *et al.*, 1995). The full length receptor is predicted to be between 445 and 448 amino acids, is located is located on chromosome 10q21-q24 (Gelernter *et al.*, 1995) and contains two introns (Ruat *et al.*, 1993; Erdmann *et al.*, 1996; Heidmann *et al.*, 1997). There are at least four splice variants of the 5-HT_7 receptor in humans, 5-$HT_{7(a)-(d)}$, but as yet expression of the 5-$HT_{7(c)}$ has not been detected (Heidmann *et al.*, 1997). They are predicted to be members of the GPCR superfamily and have consensus sequences for two N-linked glycosylation sites in the N terminus (Bard *et al.*, 1993; Lovenberg *et al.*, 1993a,b; Meyerhof *et al.*, 1993; Plasat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Tsou *et al.*, 1994), and a number of predicated protein kinase A and C phosphorylation sites in the third intracellular loop and C terminus (Bard *et al.*, 1993; Lovenberg *et al.*, 1993a,b; Meyerhof *et al.*, 1993; Plasat *et al.*, 1993; Ruat *et al.*, 1993; Shen *et al.*, 1993; Tsou *et al.*, 1994; Nelson *et al.*, 1995; Heidmann *et al.*, 1997). To date no pharmacological differences have been detected between the 5-HT_7 isoforms. The receptor is positively linked to adenylate cyclase *via* $G_{s\alpha}$ (Obosi *et al.*, 1997).

1.5 Therapeutic Roles for the 5-HT_{1A} Receptor

The 5-HT_{1A} receptor has been indicated in a wide variety of therapeutic roles, the vast majority of which are due to its CNS expression. It has been indicated in thermoregulation (Balcells-Olivero *et al.*, 1998; Seletti *et al.*, 1995), aggression (Miczek *et al.*, 1998; Hen 1996), depression (Blier *et al.*, 1997; Shiah *et al.*, 1998), anxiety (Parks *et al.*, 1998; Ramboz *et al.*, 1998) and neurogenesis (Gould 1999). It also plays a role in immune responses (Mossner and Lesch 1998).

Administration of the 5-HT_{1A} selective agonist 8-OH-DPAT decreases the core body temperature of rats (Balcells-Olivero *et al.*, 1998; Torup *et al.*, 2000). In the latter study, Torup *et al.* found that 8-OH-DPAT had a neuroprotective role following a 2-vessel occlusive ischaemic event. Core body temperature of 8-OH-DPAT treated and control rats was monitored for 24 hours post ischaemia and after seven days the number of viable CA1 neurons were counted. In the 8-OH-DPAT treated rats more than twice as many viable cells were found and it was proposed that this was due to the significantly lowered core body temperature induced by the 5-HT_{1A} agonist (Torup *et al.*, 2000).

In patients suffering from depression, treatment with 5-HT_{1A} agonists is accompanied by a decreased hypothermic response when compared to non depressed controls (Cowen *et al.*, 1994; Lesch *et al.*, 1990), indicating that an abnormality in the 5-HT_{1A} receptor or its signalling is involved. This has been widely known for many years and a number of treatments for depression are aimed at 5-HT and the 5-HT_{1A} receptor where depression is associated with an increase in the firing activity of 5-HT neurons of the dorsal raphe nucleus (Pineyro and Blier, 1999). The use of both SSRIs (selective serotonin uptake inhibitors) and MOAIs (monoamine oxidase inhibitors) for the treatment of depression are both aimed at altering the levels of 5-HT in the synaptic cleft following 5-HT neuron activation. This leads to an increased activation of the somatodendritic 5-HT_{1A} autoreceptor and the activation of a negative feedback loop that decreases the firing of the 5-HT neuron (Pineyro and Blier, 1999). The drawback to these therapeutic approaches is that following their sustained administration, the somatodendritic 5-HT_{1A} receptor desensitises and the effectiveness of the drugs decreases. This may be due to downregulation of the presynaptic 5-HT_{1A} receptor. Following 14-21 days of treatment the pre-treatment firing frequency of the 5-HT neurons returns (Dong *et al.*, 1997,1998; Pineyro and

Blier, 1999). Interestingly, there does not appear to be any effect on the postsynaptic CA_3 5-HT_{1A} mediated responses over this time (Blier and Monigny 1994). If these contrasting effects are due to receptor reserve postsynaptically and a lack of reserve presynaptically or another mechanism remains unclear (Pineyro and Blier, 1999).

The presynaptic 5-HT_{1A} autoreceptor has also been indicated as having a role in stress and anxiety (Chaouloff *et al.*, 1999). Increases in both the firing activity of 5-HT neurons and in the levels of 5-HT in the synaptic cleft of the dorsal raphe nucleus are associated with stress. A decrease in the sensitivity of somatodendritic 5-HT_{1A} autoreceptors to 5-HT is recorded following the chronic exposure to stressors, but not to acute exposure (Laaris *et al.*, 1997) that may indicate the down-regulation of the receptor. These characteristics of stress/anxiety and depression are supported by the results of behavioural studies on 5-HT_{1A} knockout mice (Ramboz *et al.*, 1998; Heisler *et al.*, 1998). Both research groups found that homozygous 5-HT_{1A} knockout mice showed increased levels of anxiety coupled with decreased levels of depression indicating that the 5-HT_{1A} receptor plays a pivotal role in the pathophysiology of these two disease states.

It has been demonstrated that serotonin has a role in the growth of developing and adult brain (Manzer *et al.*, 1997; Yan *et al.*, 1997). Specifically, the neurogenesis in adult mammalian brain dentate gyrus, an area that is rich in 5-HT_{1A} receptors, is enhanced by 5-HT receptor activation (Jacobs *et al.*, 1998). Growth in the majority of brain regions is restricted to gestation, but continues in the granule cell layer of the dentate gyrus into adulthood where the majority of new cells differentiate into mature neurons (Gould *et al.*, 1997,1998,1999a). It was suggested that these cells could be involved in growth and learning (Gould *et al.*, 1999). Conditions such as stress (Gould *et al.*, 1997), ageing (Gould *et al.*, 1999a), and NMDA receptor activation (Cameron *et al.*, 1995), that inhibit granule cell genesis also decrease the density of 5-HT fibres or 5-HT_{1A} receptors, or inhibit 5-HT release in the dentate gyrus (Gould *et al.*, 1999b). Activation of 5-HT_{1A} receptors with 8-OH-DPAT increases the rate of proliferation of granule cell precursors, the majority of which differentiate into granule neurons (Gould *et al.*, 1999b) suggesting that the 5-HT_{1A} receptor is directly involved in the stimulation of adult dentate gyrus neuronal growth.

The 5-HT_{1A} receptor may also have roles in aggression (Hen 1996), where 5-HT_{1A} agonists decreased aggressive behaviour in mice, in Tourette's syndrome (Lam

et al., 1996; Robertson 2000), where a number of mutant 5-HT_{1A} receptors have been detected in patients, in immune function (Mossner and Lesch 1998), where 5-HT_{1A} receptor antagonists inhibit the production of T_{H1}-type cytokines by memory T cells and agonists abrogate monocyte induced suppression of NK cell functions, and also in schizophrenia (Millan 2000) where D_2 receptor antagonists that have partial agonist activity at 5-HT_{1A} receptors may provide improved treatment of the disease.

1.6 G proteins

Heterotrimeric GTP binding proteins (G proteins) transduce extracellular signals that activate transmembrane spanning G protein coupled receptors to the intracellular environment *via* second messenger systems. Heterotrimeric G proteins consist of α, β, and γ subunits and transduce signals from GPCRs activated by such diverse ligands as photons, neurotransmitters and hormones to second messenger regulating systems ranging from adenylyl cyclase to ion channels and the MAP kinase signalling cascade (Figure 1.5). Currently more than 20 α subunits have been classified and have been assigned to five different families (Simon *et al.*, 1991). There are five different G protein β subunits and at least 12 different γ subunits. Initially, signalling was thought to progress through the α subunits with the βγ dimer having a membrane anchorage role. However, it has been shown that the latter is also involved in effector signalling (Clapham and Neer 1997).

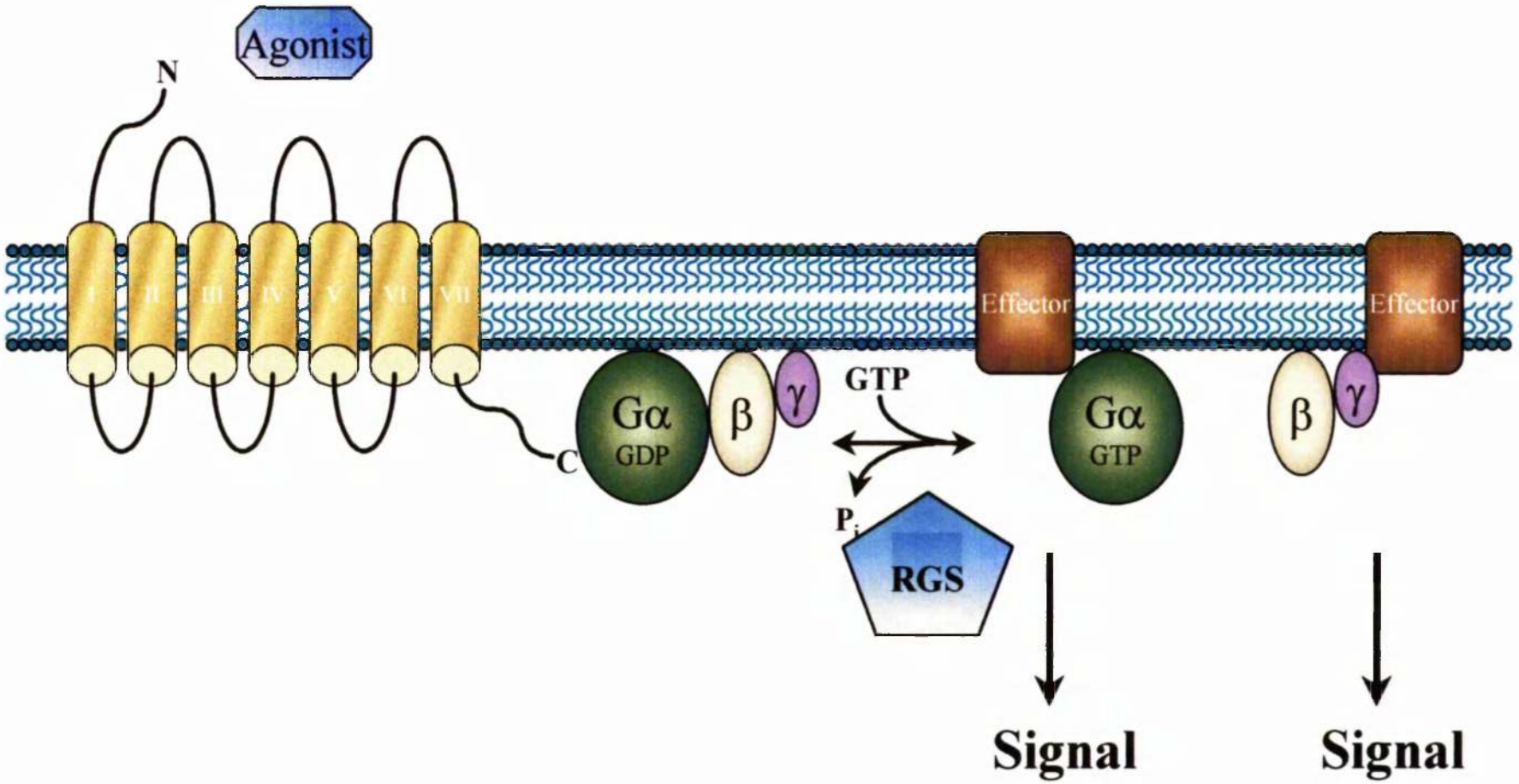
1.6.1 G protein Structural Features

The structure of $G_{t\alpha}$ and $G_{i1\alpha}$ have been solved in several conformation states including the GDP bound form (Lambright *et al.*, 1994; Coleman *et al.*, 1994; Mixon *et al.*, 1995), the GTPγS form (Coleman *et al.*, 1994; Noel *et al.*, 1993), the αβγ oligomer and the βγ dimer (Wall *et al.*, 1995; Lambright *et al.*, 1996; Sondek *et al.*, 1996) as well as the complex between $G_{i1\alpha}$ and RGS4 protein (Tesmer *et al.*, 1997). These studies have revealed a considerable amount about the structures of the α, β and γ subunits and their interactions with one another.

The α subunit has a $p21^{ras}$-like domain and an α helical domain separated by the guanine nucleotide binding site. The confomation of the α subunit with GDP bound is different to that when GTP is bound by movements in three discontiguous

Figure 1.5 GPCR – G protein signalling cascade

Following agonist activation of a GPCR, a conformational change takes place in the receptor leading to G protein binding. This interaction results in an exchange of GDP for GTP and the subsequent dissociation of the G protein from the receptor and the G protein α and $\beta\gamma$ subunits from one another. The free G protein subunits modulate the activity of effectors generating a signal within the cell. The hydrolysis of GTP to GDP leads to the re-association of the G_α and $\beta\gamma$ subunits and termination of activity.


Agonist
N
I
II
III
IV
V
VI
VII
C
Effector
Effector
Gα
GDP
β
γ
GTP
Gα
GTP
β
γ
P_i
RGS
Signal
Signal

loops, switch I that connects the helix αF and strand β2, switch II that connects α_2 and β_4, and switch III that connects α_3 and β_4. The switch II region interacts with the βγ dimer (Faurobert *et al.*, 1993; Thomas *et al.*, 1993; Conklin and Bourne 1993), with switch II and switch III participating in the formation of effector binding sites I and II which suggests that dissociation of the βγ dimer is a prerequisite for effector binding at these sites. A third effector binding site is formed by helix α_4 and β_6.

The βγ subunits provide a more rigid structure, with the β subunit consisting of a rigid propeller like core composed of seven WD40 repeats and the γ subunit composed of an extended α helical conformation. The amino termini of the β and γ subunits form a coiled coil which is farnesylated or geranylgeranylated and is oriented towards the amino terminal α helix of the α subunit (Wall *et al.*, 1995; Lambright *et al.*, 1996). The βγ dimer does not significantly change structurally following α subunit dissociation (Wall *et al.*, 1995; Lambright *et al.*, 1996), indicating an important role as a scaffold protein for the α subunit, the reassociation of which results in its deactivation.

In a simplified form the mechanism of signalling through G proteins has been well understood for a number of years (Hepler and Gilman 1992). In the basal state the G protein exists as the αβγ heterotrimer with GDP bound to the α subunit, with the slow rate of GDP release (k_{off} is 10-100 times slower than k_{cat} of GTP) maintaining the G protein in the "off" state and leading to βγ binding with high affinity. Agonist binding to a receptor leads to a conformational change in the GPCR and reveals sites on the intracellular loops of the receptor where an appropriate G protein may bind. Receptor binding leads to a structural change in the G protein α subunit and a decrease in affinity for GDP and the subsequent binding almost instantaneously of GTP. The α subunit and βγ dimer dissociate and interact with second messenger systems modulating their activity. The intrinsic GTPase activity of the α subunit cleaves the terminal phosphate group from GTP, then the α subunit returns to the inactive conformation and reassociates with and deactivates the βγ dimer.

This simple system of G protein activation and deactivation is subject to many modulations. The GTPase activity of the α subunit can be accelerated by the effector (Bernstein *et al.*, 1992) or by the RGS family of proteins (Dohlmann and

Thorner 1997; Berman and Gilman 1998) which may also modulate the signalling of the βγ dimer. Signalling is also affected by intrinsic levels of basal activity within receptors (Schutz and Freissmuth 1992; Lefkowitz *et al.*, 1993) that in the absence of agonist can result in the activation of G proteins. It is also becoming apparent that receptors and G proteins do not have to dissociate to terminate signalling and exist as a complex prior to signalling (Chidiac 1998; Rebois *et al.*, 1997). The simple "on or off" model of signalling is also unlikely, with evidence pointing towards multiple activation states of GPCRs where agonists may regulate the levels of interaction with different classes of G protein and thus effectors (Krumins and Barber, 1997; Perez *et al.*, 1996; Kenakin, 1995; Berg *et al.*, 1998).

1.6.2 G protein α Subunits

Over twenty α subunits of the G protein heterotrimer are known to exist and can be divided into a number of groups based on structure and function; the $G_{s\alpha}$ group stimulates adenylyl cyclase, the $G_{i\alpha}$ group which inhibits adenylyl cyclase, the $G_{t/g\alpha}$ group or the transducins and gustducins which stimulate retinal cGMP phosphodiesterases and presumably a related gustatory effector, the $G_{q\alpha}$ group that activates PLC_β and non receptor tyrosine kinases, and finally the $G_{12/13\alpha}$ group that regulates low molecular weight G proteins of the rho family. The α subunit in the inactive form has GDP bound and is complexed with the βγ dimer. Activation of the G protein results in the exchange of GDP for GTP and the dissociation of the α subunit from the βγ dimer and the subsequent modulation of effector activity. The intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP and inactivates the α subunit which reassociates with the βγ dimer and in turn deactivates its signalling. The wide variety of α, β and γ subunits allows for specific modulation of a large number of effectors.

1.6.2.1 $G_{i\alpha}$ proteins

There are six subtypes of "inhibitory" G protein, $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$, $G_{o1\alpha}$, $G_{o2\alpha}$ and G_z that range in size from 39-41 kDa. All are negatively coupled to adenylyl cyclase (Gilman 1987) and with the exception of G_z, can also be characterised by their sensitivity to pertussis toxin (Katada *et al.*, 1984). Pertussis toxin catalyses the

ADP ribosylation of Cys^{351}, a feature that G_z lacks, at the C terminal end of the G_α protein, a modification that prevents receptor mediated exchange of GDP for GTP and thus maintains the G protein in the inactive state. The G_i protein was first purified from rabbit liver (Bokoch *et al.*, 1983, 1984) and human erythrocytes (Codina *et al.*, 1983, 1984), and $G_{o\alpha}$, which has been estimated to make up 0.5% of brain membrane proteins (Offermans 2001), from brain tissue (Sternweis and Robishaw 1984; Neer *et al.*, 1984). This group of G proteins also couple to inhibition of neuronal Ca^{2+} channels and stimulation of K^+ channels through release of the $\beta\gamma$ dimer. Members of this family are targets for N-myristoylation, the attachment of myristate through an amide bond to a glycine residue at a protein N terminus, a reaction catalysed by N-myristol coA-transferase (NMT) (Johnson *et al.*, 1994). This requires a Gly^2 and usually Ser^6/Thr^6 motif. Although insufficient for stable membrane attachment (Peitzsch and McLaughlin 1993), this modification was found to be a pre-requisite for palmitoylation (Mumby *et al.*, 1994; Hallak *et al.*, 1994; Galbiati *et al.*, 1994). The attachment of palmitate by a thioester bond to a cysteine residue near the N terminus of the G_α protein is reversible and no clear consensus sequence or catalysing enzyme have been identified (Chen and Manning 2001). The modification can occur at the plasma membrane, an area established as having the capacity for palmitoylation (Dunphy *et al.*, 1996), following weak attachment of the protein by myristoylation. It alone is sufficient to stably anchor the G_α subunit to the membrane (Shahinian and Silvius 1995). Interaction of the G_α subunit with the $\beta\gamma$ dimer also enhances palmitoylation *in vitro* (Dunphy *et al.*, 1996).

The myristate and palmitate modifications have been shown to considerably increase the affinity of $G_{i\alpha}$ subunits for the $\beta\gamma$ dimer (Jones *et al.*, 1990; Linder *et al.*, 1991), with the interaction between $G_{o\alpha}$ and $\beta\gamma$ dimers being unstable and the $\beta\gamma$ dimer being unable to suppress GDP dissociation. The effects on $G_{i\alpha}$ proteins are not as pronounced, but pertussis toxin catalysed ADP ribosylation of the α subunit is reduced seven fold in the absence of myristoylation (Jones *et al.*, 1990; Linder *et al.*, 1991).

Myristoylation has been shown to be a pre-requisite for $G_{i\alpha}$ inhibition of adenylyl cyclase *in vitro* (Taussig *et al.*, 1993). In Sf9 membranes, a myristoylated $G_{i\alpha}$ protein from *E.coli* inhibited adenylyl cyclase, but a deficient protein did not. In

rat 1a cells, a similar lack of adenylyl cyclase inhibition has been recorded, but the effect of lack of palmitoylation was not investigated (Gallego *et al.*, 1992).

The 5-HT_{1A} receptor has been shown to promote palmitate exchange on endogenous $G_{i\alpha}$ proteins in CHO cells (Chen and Manning 2000). Pulse chase experiments with [^{3}H]-palmitate demonstrated that agonist activation by 8-OH-DPAT resulted in agonist concentration and time dependent increase in $G_{i\alpha}$ protein [^{3}H]-palmitate radiolabelling. These effects were inhibited by the antagonist MPPI and pertussis toxin treatment, demonstrating the requirement for receptor activation.

The $G_{z\alpha}$ protein was shown to be rapidly phosphorylated following the incubation of human platelets with a PKC-activating phorbol ester (Carlson *et al.*, 1989) and this was suggested to occur at either Ser^{25} or Ser^{27} (Lounsbury *et al.*, 1991). Subsequent studies identified Ser^{27} as a preferred site of phosphorylation with Ser^{16} also being targeted (Lounsbury *et al.*, 1993). It is unclear if the activation state of the $G_{z\alpha}$ subunit affects phosphorylation, but it is clear that the interaction of the $\beta\gamma$ dimer inhibits phosphorylation (Fields and Casey, 1995; Kozasa and Gilmann 1996; Wang *et al.*, 1999). The converse is also true, with phosphorylation inhibiting the binding of $G_{z\alpha}$ with the $\beta\gamma$ dimer (Fields and Casey, 1995; Kozasa and Gilmann 1996; Wang *et al.*, 1999). Data for the other members of this family are not yet consistent with some indicating PKC or PMA induced phosphorylation (Daniel-Issakani *et al.*, 1989; Strassheim and Malbon, 1994) and others indicating a lack of phosphorylation (Kozasa and Gilmann 1996).

The intrinsic GTPase activity of this family of G_α subunits can be increased by a family of proteins know as Regulators of G proteins Signalling (RGS) proteins (Dohlmann and Thorner 1997; Berman and Gilman 1998). These proteins bind to the GTP bound form of G_α subunits and stabilise the transition state resulting in an increase in k_{cat} for GTP hydrolysis. This family of proteins will be discussed further in section 1.5.

A large number of G protein α subunit knockout mice have been generated to analyse G protein function in the intact organism. Knockout mice for the $G_{i\alpha}$, $G_{o\alpha}$ and $G_{z\alpha}$ have been generated (Valenzuela *et al.*, 1997; Jiang *et al.*, 1998; Yang *et al.*, 2000; Hendry *et al.*, 2000;Rudolph *et al.*, 1995). $G_{o\alpha}$ (-/-) mice showed no gross morphological abnormalities and neural pathfinding and growth cone collapse was normal (Valenzuela *et al.*, 1997). However, they were smaller and weaker than their

littermates and had greatly reduced postnatal survival rates (Valenzuela *et al.*, 1997; Jiang *et al.*, 1998). These mice suffered from tremors, had occasional seizures and showed elevated motor activity and extreme turning behaviour. Inhibition of Ca^{2+} channel currents in dorsal root ganglia following opioid receptor activation was reduced by about 30% (Jiang *et al.*, 1998). $G_{z\alpha}$ (-/-) mice in some cases showed more pronounced increases in motor activity compared to WT following cocaine administration (Yang *et al.*, 2000) but no alteration in the acute effects of morphine in a different strain (Hendry *et al.*, 2000). The effects of catecholamine re-uptake inhibitors on behaviour were abolished (Yang *et al.*, 2000). $G_{i2\alpha}$ deficient mice develop a diffuse inflammatory bowel disease and many show colonic adrenocarcinomas (Rudolph *et al.*, 1995).

1.6.2.2 $G_{s\alpha}$ proteins

There are four splice variant from the same gene encoding the $G_{s\alpha}$ protein of which two major isoforms are expressed, one of 45 kDa and another of 52kDa (Northrup *et al.*, 1980; Sternweis *et al.*, 1981; Hildebrandt *et al.*, 1984). The protein is characterised by is ability to stimulate adenylyl cyclase activity and also by cholera toxin catalysed ADP ribosylation that results in the inactivation of its GTPase activity resulting in persistent activation (Bourne *et al.*, 1991). The $G_{s\alpha}$ protein is not myristoylated but is palmitoylated at a single site near the N terminus. It was shown in $G_{i\alpha}$ proteins that myristoylation is a pre-requisite for palmitoylation but no equivalent lipid modification has been identified for the $G_{s\alpha}$ protein. Circumstantial evidence that a postranslational, as yet unidentified modification, does occur (not palmitoylation) comes from effects of $G_{s\alpha}$ to activate adenylyl cyclase (Kleuss and Gilman, 1997). The EC_{50} of $G_{s\alpha}$ purified from rabbit liver for activation of adenylyl cyclase was about 0.1nM but for $G_{s\alpha}$ expressed and purified from *E.coli* it was 50nM (Kleuss and Gilmann, 1997). This difference was lost following removal of 30 amino acids from the N terminus of the $G_{s\alpha}$ protein and partitioning experiments suggested that the modification was hydrophobic (Kleuss and Gilman, 1997).

In pulse chase experiments with the β_2 adrenergic receptor, an increase in $[^3H]$-palmitate incorporation to the $G_{s\alpha}$ protein was measured (Wedegaertner and Bourne 1994; Mumby *et al.*, 1994). The degree of incorporation to receptor – $G_{s\alpha}$ fusion proteins correlated with the intrinsic efficacy of agonists to stimulate adenylyl

cyclase (Loisel *et al.*, 1999). Phosphorylation of the $G_{s\alpha}$ protein in response to PKC stimulation (Lounsbury *et al.*, 1993) or PKA (Wang *et al.*, 1999) has not been recorded.

Homozygous knockout mice embryos for the $G_{s\alpha}$ protein do not survive beyond day 10 (Yu *et al.*, 1998). Heterozygous mice do survive and show increased birth weight with subcutaneous oedema, later exhibiting delayed development of thymus, kidney and cerebellum and ataxia and tremors (Yu *et al.*, 1998). Most animals die during the early postnatal weeks and are resistant to parathyroid hormone (PTH).

1.6.2.3 $G_{t/g\alpha}$ proteins

Activation of the light sensitive GPCR rhodopsin results in interaction with G_t protein, transducin (Gilman, 1987). There are two forms of this protein, $G_{t1\alpha}$ and $G_{t2\alpha}$, one which is present exclusively in cone photoreceptor outer segments, $G_{t2\alpha}$ (Lerea *et al.*, 1986) and one which is present in the rod outer segments, $G_{t1\alpha}$ (Grunwald *et al.*, 1986). These proteins activate cGMP phosphodiesterases. Gustducin, or $G_{gust\alpha}$ protein is thought to be responsible for the mediation of "bitter" taste and is assumed to modulate phosphodiesterase activity in a similar manner to transducin (Kolesnikov and Margolskee 1995; Mclaughlin *et al.*, 1992; Ming *et al.*, 1998; Ruiz-Avila *et al.*, 1995; Spielman 1998; Wong *et al.*, 1996; Yan *et al.*, 2001). It was suggested that the γ subunit that interacts with $G_{gust\alpha}$ is γ_{13} as it has been shown to colocalise with the G_α subunit (Huang *et al.*, 1999). The $G_{gust\alpha}$ protein has also been shown to colocalise with the novel family of 7TM bitter taste receptors (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000).

Heterozygous knockout mice for the $G_{t1\alpha}$ protein appear to be largely normal, with homozygotes demonstrating defective light responses and developing mild retinal degeneration with age (Clavert *et al.*, 2000). $G_{gust\alpha}$ knockout mice show decreased electrophysiological and behavioural responses to bitter and sweet agents, indicating a role for $G_{gust\alpha}$ in the perception of "sweet" stimuli also, but responses to sour and salty stimuli were identical to those of WT mice (Lindemann 1996; Kinnamon and Margolski 1996).

1.6.2.4 $G_{q\alpha}$ proteins

There are five members of the $G_{q\alpha}$ family of α subunits, $G_{q\alpha}$ itself, $G_{11\alpha}$, $G_{14\alpha}$, $G_{15\alpha}$ and $G_{16\alpha}$ (Pang and Sternweis 1990). These proteins are pertussis toxin insensitive, lacking the Cys residue that the toxin targets for ADP ribosylation. They are positively coupled to PLC-β and have lower rates of GTPase activity that those of the $G_{i\alpha}$ and $G_{s\alpha}$ proteins (Fields and Casey 1997). $G_{q\alpha}$ and $G_{11\alpha}$ are fairly ubiquitously expressed (Fields and Casey 1997), with $G_{14\alpha}$ expressed predominantly in spleen, lung, kidney and testis (Wilkie *et al.*, 1991) and $G_{15\alpha}$ and $G_{16\alpha}$ expressed in cells of myeloid and lymphoid lineage (Wilkie *et al.*, 1991; Amatruda *et al.*, 1991). The members of this family are not myristoylated but are palmitoylated (Chen and Manning 2001), and similar to $G_{s\alpha}$, lipid modifications other than palmitoylation is suspected (Hepler *et al.*, 1996). It has also been suggested that interaction with the $\beta\gamma$ dimer is a pre-requisite for palmitoylation, as mutants unable to bind $G_{\beta\gamma}$ were not palmitoylated (Evanko *et al.*, 2000). A modification that allowed myristoylation restored palmitoylation, but not $G_{\beta\gamma}$ interaction and it was suggested that $G_{\beta\gamma}$ acted for $G_{q\alpha}$ in the same way as myristoylation does for $G_{i\alpha}$ (Evanko *et al.*, 2000). The incorporation of palmitate into $G_{q\alpha}$ proteins increases in response to receptor activation, both with serotonin receptors and α-adrenergic receptors *in vitro* (Bhamre *et al.*, 1998; Gurdal *et al.*, 1997), as does tyrosine phosphorylation following carbachol activation of the M_1 muscarinic receptor (Umemori *et al.*, 1997).

$G_{q/11\alpha}$ knockout mice die at embryonic day 11 due to severe thinning of the myocardial layer of the heart (Offermans *et al.*, 1998), with both the trabecular ventricular myocardium and the subepicardial layer being underdeveloped. The receptors involved in the activation of $G_{q/11\alpha}$ during embryonic development are unknown. Knockout mice of $G_{15\alpha}$, the murine equivalent of $G_{16\alpha}$, showed no cellular defects apart from a reduced effect of C5a in macrophages (Davignon *et al.*, 2000), showing normal hematopoiesis and normal morphology and function of major immunological tissues. This may be surprising as the $G_{15\alpha}$, and the human $G_{16\alpha}$, are exclusively expressed in hemapoietic cells (Amatruda *et al.*, 1991; Wilkie *et al.*, 1991).

1.6.2.5 $G_{12/13\alpha}$ proteins

This family of G protein α subunits are involved in the activation of the rho family of low molecular weight G proteins that affect cytoskeletal structure (Prasad *et al.*, 1995; Buhl *et al.*, 1995). Mutationally activated $G_{12\alpha}$ and $G_{13\alpha}$ proteins have been implicated in the induction of mitogenesis and neoplastic transformation in NIH3T3 and Rat-1 cells (Xu *et al.*, 1993; Voyno-Yasenetskaya *et al.*, 1994). In contrast to this is their involvement in the induction of apoptosis and JNK mediated by two MAPKK kinases, MEKK1 and ASK1 (Berestetskaya *et al.*, 1998). This apoptosis could be inhibited by Bcl-2 (Berestetskaya *et al.*, 1998). Neither $G_{12\alpha}$ nor $G_{13\alpha}$ are myristoylated but they are palmitoylated, with $G_{12\alpha}$ having one potential site and $G_{13\alpha}$ having two (Chen and Manning, 2001). Both $G_{12\alpha}$ and $G_{13\alpha}$ are phosphorylated, a process that can be induced for $G_{12\alpha}$ following exposure of NIH3T3 cells to the PKC activator, PMA, most likely at Ser^{38}, a site that strongly resembles Ser^{16} in $G_{z\alpha}$ (Kazasa and Gilmann 1996). The situation for $G_{13\alpha}$ is less clear. It also appears to be a substrate for PKC mediated phosphorylation but possibly requires different conditions (Kozasa and Gilmann, 1996; Offermanns *et al.*, 1996).

$G_{13\alpha}$ knockout mice terminate about midgestation and show defects in the vascular system, most prominently in the yolk sac and the head mesenchym (Offermanns *et al.*, 1997) with angiogenesis including remodelling of existing endothelial cells being severely disrupted even in the presence of $G_{12\alpha}$ expression. $G_{12\alpha}$ knockout mice showed no obvious defects.

1.6.3 G protein βγ Subunits

The other members of the G protein heterotrimer are the β and γ subunits. Initially seen as only a docking protein for G_α subunits it has been known for some time that release of the $G_{\beta\gamma}$ dimer following receptor activation results in the modulation of a variety of effector proteins (Clapham and Neer 1997). There are known to be 5 G protein β subunits and at least 12 γ subunits (Holler *et al.*, 1999) making for a very large number of possible G protein αβγ trimer combinations. The $G_{\beta\gamma}$ dimer functionally acts as a monomer as the two subunits cannot be separated under nondenaturing conditions (Holler *et al.*, 1999). The βγ dimer has been shown,

in the presence of activated $G_{s\alpha}$, to increase the activity of type II adenylyl cyclase isoforms (Taussig *et al.*, 1993; Sunahara *et al.*, 1996) and inhibit type I-like adenylyl cyclase as well as stimulate PLC-β (Camps *et al.*, 1992, 1993; Smrcka and Sternweis 1993). The $G_{\beta\gamma}$ dimer can also activate G protein regulated inward rectifying K^+ (GIRK) channels (Wickman *et al.*, 1994) and inhibit neuronal Ca^{2+} channels (Herlitze *et al.*, 1996, 1997; Zamponi *et al.*, 1997). In a less defined mechanism they can also activate MAP kinase (Crespo *et al.*, 1994; Van Biesen *et al.*, 1995; Lopez-Ilasaca *et al.*, 1997). The $G_{\beta\gamma}$ dimer has a role in the regulation of receptor desensitisation, binding and redistributing G protein receptor kinases (GRKs) at the membrane (Premont *et al.*, 1995; Lohse 1993) and activating dynamin I, a GTPase protein involved in the pinching off of clathrin coated vesicles from the plasma membrane (Lin and Gilmann 1996). The dimer has also been shown to activate the nonreceptor tyrosine kinases Btk and Tsk (Langhans-Rajasekaran *et al.*, 1995).

The association of the G protein α subunit and the βγ dimer has two functions. The βγ dimer maintains the G_α subunit in a conformation that slows the k_{off} rate for GDP release and maintains the protein in an inactive state (Holler *et al.*, 1999). The G_α subunit has the same effect on the βγ dimer. Following GTP hydrolysis, the G_α subunit returns to a structural confomation that favours $G_{\beta\gamma}$ binding and thus deactivates $G_{\beta\gamma}$ modulation of effector proteins (Holler *et al.*, 1999).

The G_β subunits are approximately 340 amino acids in length with predicted molecular weights between 35 and 39 kDa and share a high degree of homology with each other (Clapham and Neer 1997). Not all G_β and G_γ protein combinations appear to be possible. While $G_{\beta1}$ can combine with all of the known G_γ subunits, the $G_{\beta2}$ protein cannot combine with $G_{\gamma1}$ (Clapham and Neer 1997).

The G protein βγ dimer is modified post-translationally. The $G_{\gamma12}$ subunit is a substrate for PKC both *in vitro* and in intact cells (Morishita *et al.*, 1995; Yasuda *et al.*, 1998). The $G_{\gamma12}$ subunit is the only member of the γ subunit family to contain a SSK motif near its N terminus, the first serine of which is the proposed site of phosphorylation, that is phosphorylated by both PKCα and β, less well by δ and ε, and not at all by ζ *in vitro* (Morishita *et al.*, 1995; Yasuda *et al.*, 1998). This modification was found to increase the affinity of the $G_{\beta\gamma12}$ dimer for $G_{o\alpha}$ and also $G_{i\alpha}$ (Morishita *et al.*, 1995), that lead to an increase in high affinity receptor binding

(Yasuda *et al.*, 1998). It was also found that this phosphorylation lead to a decrease in stimulation of adenylyl cyclase type II but had no effect on the activity of PLC-β (Yasuda *et al.*, 1998).

Other modifications that have been reported include prenylation. G_γ subunits contain a CAAX motif near the C terminus that directs this modification, but the form of prenylation varies, with $G_{\gamma 1}$ being farnesylated and $G_{\gamma 2}$ being geranylgeranlyated (Clapham and Neer, 1997). Removal of the Cys residue that is the target for modification results in mutant $G_{\beta\gamma}$ dimers not being properly targeted to the plasma membrane and being found in the cytosol (Muntz *et al.*, 1992; Spiegel *et al.*, 1991) indicating a membrane anchorage role for the prenylation.

$G_{\beta\gamma}$ subunits enhance the interaction between receptors and G_α subunits and have been shown to bind directly to receptors, for example β-adrenergic receptors and rhodopsin (Heither *et al.*, 1992; Philips and Cerione 1992; Philips *et al.*, 1992; Higashijima *et al.*, 1987). The composition of the βγ dimer also effects the interaction of G_α subunits with receptors. $G_{\beta 1}$ with $G_{\gamma 1}$ can support the binding of $G_{t\alpha}$ and rhodopsin, but $G_{\beta 1\gamma 2}$ cannot (Kisselev *et al.*, 1995). So far there have been no reports of either G_β of G_γ subunit knockout mice (Offermanns *et al.*, 2001).

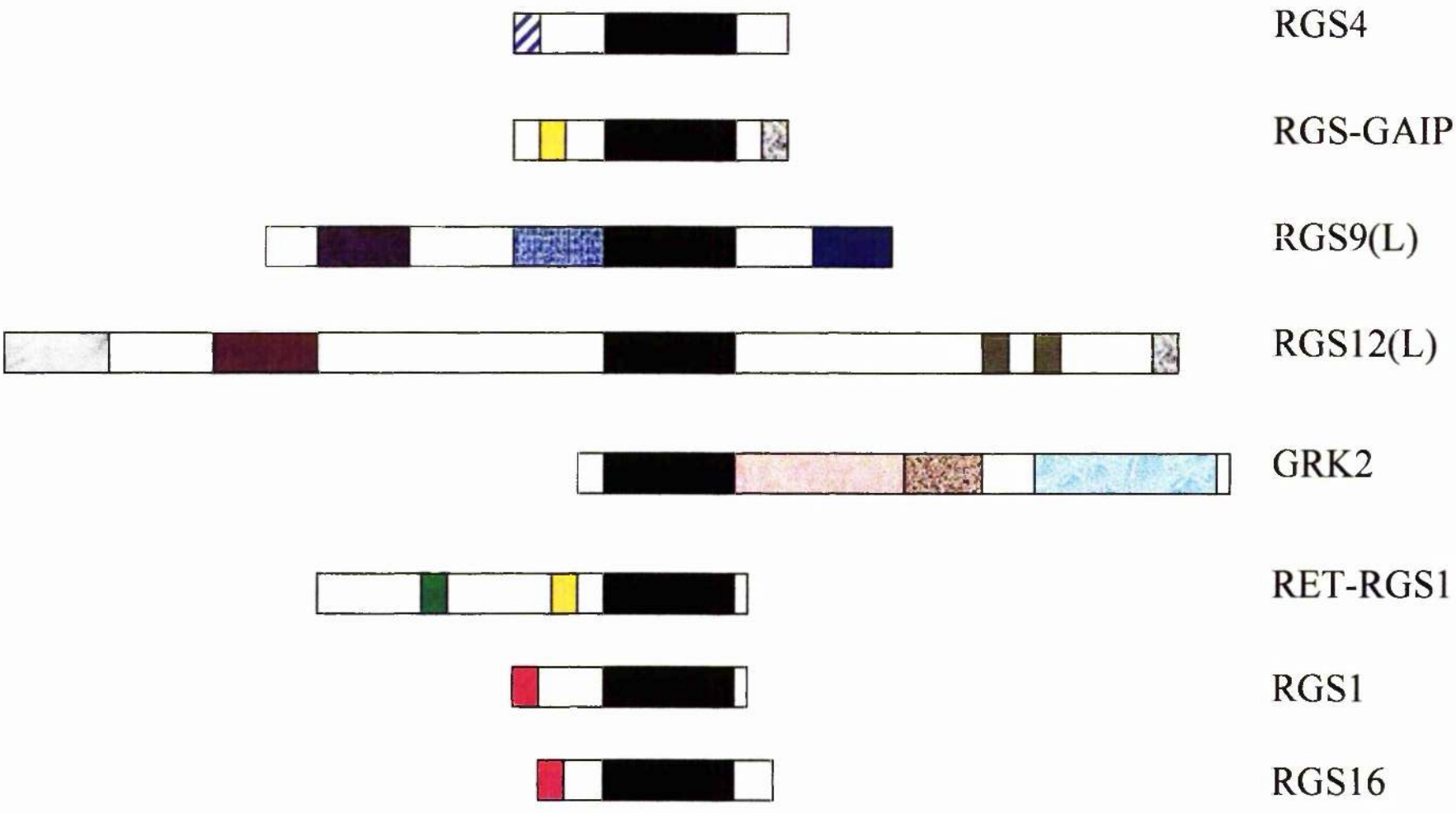
1.7 Regulators of G protein Signalling (RGS) Proteins

It was thought that stimulation of GPCRs involved agonist binding a receptor that bound a G protein, the activation of which resulted in the modulation of a second messenger system. Recently a fourth component of this signalling cascade was identified, the regulators of G protein signalling (RGS) proteins (Dohlman and Thorner 1997; Koelle 1997; Berman and Gilman 1998). These proteins add another level of modulation by binding to and increasing the intrinsic level of GTPase activity of the G protein α subunit. Initially these proteins were recognised in lower eukaryotes as negative regulators of G protein signalling (Dohlman *et al.*, 1996; Yu *et al.*, 1996; Koelle and Horvitz, 1996). However, with a family of mammalian RGS proteins of nearly 30, these proteins have been found to fulfil roles other than as GTPase activating proteins (GAPs) (Hepler, 1999).

The common structural feature of all RGS proteins is an approximate 125 amino acid RGS domain (Berman and Gilman, 1998; Hepler, 1999; De Vries *et al.*,

Figure 1.6 Structural Features of RGS Proteins

All RGS proteins contain an approximate 120 amino acid RGS domain and some members contain other structural features including: PDZ domain (PSD-95, disc-large and zo-1), DEP domain (dishevelled, egl-10 and pleckstrin), GGL domain (G protein gamma subunit like), PTB domain (phosphotyrosine binding), GRK domain (G protein coupled receptor kinase) and $G_{\beta\gamma}$ binding (G protein $\beta\gamma$ subunits).


RGS4
RGS-GAIP
RGS9(L)
RGS12(L)
GRK2
RET-RGS1
RGS1
RGS16

2000; Burchett, 2000). The domain is responsible for the GTPase activating property of the proteins, but within the family it has only between 45-80% sequence homology (Burchett, 2000). The RGS domain contains three distinct highly conserved GH (GAIP or GOS Homology) domains that in mammalian RGS proteins are nearly contiguous (DeVries *et al.*, 1995; Druey *et al.*, 1996; Koelle and Horvitz, 1996). The proteins can also act as G protein antagonists, inhibiting the binding of the G_α proteins to its effector systems (Hepler *et al.*, 1997; Tesmer *et al.*, 1997). In some cases this may actually prolong the signalling of $G_{\beta\gamma}$ dimers by preventing their reassociation with inactive $G_{\alpha\text{-GDP}}$ (Bunemann and Hosey 1998).

RGS proteins bind to the activated GTP bound form of G_α (Hepler *et al.*, 1997; Tesmer *et al.*, 1997; Popov *et al.*, 1997), the structural features of which were investigated by determination of the crystal structure of RGS4 protein bound to $G_{i1\alpha}$ with a stable GTP substitute ($GDP\text{-}AlF_4^-$) in the GDP/GTP binding pocket (Tesmer *et al.*, 1997). The RGS domain of RGS4 was found to form nine α-helices that fold into two subdomains with the hydrophobic amino acids at the core of these domains being involved in structural stability and GAP activity (Tesmer *et al.*, 1997; Srinivasa *et al.*, 1998). Interaction between the RGS domain and the G_α protein occurred at three sites necessary for GDP/GTP and effector binding (Burchett, 2000). The amino acids Thr^{182} and Gly^{183} on $G_{i1\alpha}$ are essential for high affinity G_α-RGS binding (Tesmer *et al.*, 1997; Dibello *et al.*, 1998). Thr^{182} is conserved in the $G_{i\alpha}$ and $G_{q\alpha}$ G protein families and not in $G_{s\alpha}$ or $G_{12\alpha}$ which may explain why so far no interactions between RGS proteins and the latter two G_α protein families have been recorded (Burchett *et al.*, 2000). The preference for binding to the GTP bound form of the G_α protein suggests that the RGS protein stabilises the G_α formation required for GTP hydrolysis (Berman *et al.*, 1996; Srinivasa *et al.*, 1998), and this interaction may also have a stabilising effect on the RGS protein (de Alba *et al.*, 1999). Deletions of small portions of the RGS domains of RGS4, RGS10 or GAIP abolish their GAP activity *in vitro* (Faurobert and Hurley, 1997; Popov *et al.*, 1997; Srinivasa *et al.*, 1998).

A large degree of promiscuity of RGS protein for G_α is a feature of *in vitro* studies on this interaction. For example, $G_{i\alpha}$ proteins have been shown to interact with RGS1, RGS3, RGS4, RGS5, RGS10, RGS12, RGS16, RET-RGS1 and RGS-GAIP *in vitro* (Hunt *et al.*, 1996; Watson *et al.*, 1996; Chen *et al.*, 1997; Faurobert

and Hurley, 1997; DeVries *et al.*, 1995; Berman *et al.*, 1996; Hepler *et al.*, 1997; Snow *et al.*, 1998). Interactions of RGS proteins and other G_α families have been demonstrated with $G_{q\alpha}$ interacting with RGS2, RGS4 and RGS-GAIP (DeVries *et al.*, 1995; Berman *et al.*, 1996; Hepler *et al.*, 1997; Heximer *et al.*, 1997) and $G_{t\alpha}$ interacting with RGS9 and RGS9S (He *et al.*, 1998).

As well as the GAP activity of the RGS proteins modulating the effector signalling of the G_α subunits, effects on $G_{\beta\gamma}$ dimer signalling have been recorded. Expression of RGS proteins in mammalian cell lines attenuates ERK and p38-MAPK signalling by the $G_{\beta\gamma}$ dimer released from $G_{i\alpha}$ or $G_{q\alpha}$ activation by accelerating the reassociation of the G_α and $G_{\beta\gamma}$ subunits (Buckbinder *et al.*, 1997; Huang *et al.*, 1997; Ingi *et al.*, 1998; Zhang *et al.*, 1999). The $G_{\beta\gamma}$ dimer can also modulate the activity of GIRK and N-type Ca^{2+} channels. The inhibition of N-type Ca^{2+} channel opening by $G_{\beta\gamma}$ is attenuated in the presence of RGS proteins (Melliti *et al.*, 1999). In contrast, the effects of RGS proteins on $G_{\beta\gamma}$ dimer activation of GIRK channels is not as clear. The expression of a number of RGS proteins results in the acceleration of both GIRK channel activation and deactivation independent of a reduction in peak current (Doupnik *et al.*, 1997; Saitoh *et al.*, 1997, 1999; Kovoor *et al.*, 2000). If the only role of the RGS protein was as a G_α subunit GAP protein then both the activation and peak current would be diminished due to the accelerated reassociation of G_α and $G_{\beta\gamma}$. The acceleration of the activation rate has been speculated to be either due to acceleration of the dissociation of activated G_α from $\beta\gamma$, or by antagonising the reassociation of G_α and $\beta\gamma$ (Burchett *et al.*, 2000).

The RGS domain is what groups these proteins into a family but many of the proteins have large N and C terminal domains that fulfil other cellular functions. A few RGS proteins contain little other than the RGS domain, e.g. RGS1, RGS2 and RGS4. Others have further functional domains, like the GRKs (G protein receptor kinases) that contain a kinase domain and $G_{\beta\gamma}$ binding domain, or RGS proteins 6, 7, 9 and 11 that contain both DEP (dishevelled, Egl-10, pleckstrin) and GGL (G protein gamma like) domains (Hepler, 1999). The roles of GRK protein kinase and $G_{\beta\gamma}$ binding domains in the desensitisation of GPCR signalling have been clearly defined (Krupnik and Benovic, 1998) but their more recently discovered RGS domain, that would appear to complement their primary function, has yet to be characterised functionally. The role of the DEP domain that is found in a large

number of unrelated proteins is unclear (Ponting and Bork, 1996), but is predicted to be involved in targeting DEP containing proteins to GPCRs. For RGS proteins that do not contain traditional membrane anchorage modification such as palmitoylation or myristoylation, the DEP domain may allow targeting to the membrane and specificity of interaction by binding only to specific DEP binding proteins. For example, both RGS7 and RGS9S, which lack membrane spanning domains or membrane associated modifications, complex with the G protein β_5 subunit (Khawaja *et al.*, 1999; Watson *et al.*, 1994; Cowan *et al.*, 1998).

The GGL domains of RGS proteins also appear to have a role in specificity of protein interactions. RGS proteins 6, 7, 9 and 11 contain GGL domains that interact strongly with G_β subunits (Snow *et al.*, 1998), particularly with $G_{\beta 5}$. Both the RGS proteins 6, 7, 9 and 11 and $G_{\beta 5}$ are either exclusively or highly expressed in the brain and retina (Gold *et al.*, 1997; Granneman *et al.*, 1998; He *et al.*, 1998; Snow *et al.*, 1998; Burchett, 2000). The co-expression of $G_{\beta 5}$ and either RGS7 or RGS9 in *Xenopous* oocytes showed increased activation kinetics of GIRK channels over expression of either RGS protein or $G_{\beta 5}$ alone (Kovoor *et al.*, 2000), but it is unclear if an interaction between these proteins was involved.

Other methods of RGS protein interaction with the plasma membrane have been indicated. RGS1 contains three motifs for possible myristoylation in its N terminus (Denecke *et al.*, 1999) that could result in weak anchorage of the protein to the membrane, but further modification such as palmitoylation would be required for stable anchorage (Resh, 1999). A number of RGS proteins (RET-RGS1, GAIP and RGS17) contain cysteine "rich" regions that could be targeted for palmitoylation (DeVries *et al.*, 1996; Faurobert and Hurley, 1997; Wang *et al.*, 1998; Zheng *et al.*, 1999). RGS-GAIP is palmitoylated in this region and it has been suggested that the modification may play a role in membrane anchoring of the protein to clathrin coated vesicles where RGS-GAIP was found to localise (DeVries *et al.*, 1996).

Palmitoylation also occurs on Cys residues in regions rich in basic amino acids. RGS4, RGS5 and RGS16 all contain such regions and two cysteine residues, Cys^2 and Cys^{12} (Burchett, 2000). RGS4 is palmitoylated at these residues and is a membrane anchored RGS protein, however palmitoylation deficient mutants are still membrane anchored and have GAP activity (Srinivasa *et al.*, 1998). RGS16 is also palmitoylated at Cys2 and Cys^{12} and again this is not essential for membrane

association (Chen *et al.*, 1999; Druey *et al.*, 1999), but palmitoylation deficient mutants in this case decrease the GAP activity of the protein (Druey *et al.*, 1999).

RGS proteins have been indicated as having roles in a number of disease states. The inactivation of p53 is a common defect in human cancers and in colon carcinoma cells the expression of p53 suppresser protein is associated with an upregulation in RGS16 (Buckbinder *et al.*, 1997). Levels of RGS2 mRNA have been found to be markedly upregulated in the CNS following seizures in models of epilepsy (Ingi *et al.*, 1998). Other RGS proteins may also have roles, such as RGS7 in autosomal-dominant polycystic kidney disease (Kim *et al.*, 1999) and axin in familial adenomous polyposis (Kishida *et al.*, 1998; Behrns *et al.*, 1999). Regulation of GPCR –RGS protein interaction may have a significant role in many future therapeutic regimens.

1.8 Project Aims

As has been indicated a more complete understanding of the 5-HT_{1A} receptor and its regulation of the signal transduction mechanisms that it couples to could help in the development of new therapeutic regimes for a variety of diseases. To this end the aims of this project were fourfold: (1) to characterise the regulation of adenylyl cyclase by 5-HT_{1A} receptor-G protein fusions, (2) to determine the role of Cys^{351} mutations in the affinity of agonist activated 5-HT_{1A} receptor-G proteins interactions, (3) to examine the effects of a range of concentrations of RGS1 and RGS16 proteins on the high affinity GTPase activity of membranes expressing the 5-HT_{1A} receptor-G protein fusions, (4) to characterise the contribution of RGS proteins in the design of a robust high affinity GTPase assay for ligand characterisation using the 5-HT_{1A} receptor-G protein fusions.

Chapter 2

Materials and Methods

Materials and Methods

2.1 Materials

The materials used were obtained from the following suppliers:

Alexis Corporation, San Diego, CA, USA

DTT

American Tissue Culture Collection, Rockville, USA

Human embryonic kidney (HEK293) cells

Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK

[^{3}H]-WAY100635, [^{3}H]-8-OH-DPAT, [^{3}H]-Adenine, Glutathione Sepharose® 4B, full range Rainbow™ molecular weight marker, anti rabbit Ig horseradish peroxidase linked antibody (from Donkey)

BDH Chemicals Ltd., Poole, UK

NaCl, $MgCl_2$, acetic acid, KOH, $CaCl_2$, $MnCl_2$

BioWhittaker Molecular Applications, Rockland, ME, USA

SeaKem® LE Agarose

Camlab Ltd., UK

Plastic binding tubes PS/T400-3A

Corning, Horseheads, NY 14831, USA

5,10 and 25ml graduated sterile tissue culture plastic pipettes, pyrex borosilicate glass binding tubes

Costar, Cambridge, MA, USA

75cm^2 and 150cm^2 tissue culture flasks, 60mm and 100mm tissue culture dishes, 6,12 and 24 well tissue culture plates, cryovials

Eppendorf, Hamburg, Germany

96 Well Deepwell plates

Fisher Scientific, Loughborough, Leicestershire, UK

HEPES, EDTA, DMSO, methanol, ethanol, concentrated HCl

GIBCO BRL Life Technologies, Paisley, UK

LipofectAMINE, OptiMEM, Glutamine, BL21-SI Competent Cells

Invitrogen BV, Groningen, Netherlands

NuPAGE® Novex high-performance pre-cast gels

Iwaki, Scitech Division, Asahi, Japan

75cm^2 and 150cm^2 tissue culture flasks, 60mm and 100mm tissue culture dishes, 6,12 and 24 well tissue culture plates

Konica, Tokyo, Japan

X-ray film

NEN Life Science Products Inc., Boston, USA

[^{3}H]-MPPF, [^{32}P]-GTP

Novagen, Madison, WI, USA

Benzonase® Nuclease, bugbuster™ protein extraction reagent

Oxoid Ltd., Hampshire, UK

Tryptone, yeast extract

Packard Instruments BV, Netherlands

Ultima Gold XR liquid scintillation cocktail, Optiplate™ 96 well plates, microscint™

Pierce, Illinois, USA

SuperSignal® Chemiluminescent Substrate, Coomassic® protein assay reagent

Premier Brands UK Ltd., Merseyside, UK

Marvel

Promega, Southampton, UK

Wizard™ Miniprep Kit, restriction enzymes, G-418 sulphate

Qiagen, Crawley, West Sussex

Qiagen® plasmid maxiprep kit.

Roche Molecular Biochemicals/Boehringer-Mannheim, Germany

App[NH]p, creatine phosphate, creatine kinase, GTP, tris, bovine serum albumin fraction V, DNA molecular weight marker X (0.07-12.2kbp), restriction enzymes, IPTG

Schleicher and Schuell, Dassel, Germany

Protran® nitrocellulose transfer membrane

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

DMEM, newborn calf serum, trypsin, dowex AG50 W-X4 (200-400mesh), imidazole, forskolin, 5-HT, WAY 100635, ampicillin, ouabain, pertussis toxin, DMSO, NaCL, trichloroacetic acid, coomassie blue, IBMX, activated charcoal, ATP, cAMP, ascorbic acid, alumina, glutathione, bicinchoinic acid, Tween20

Tocris, Bristol, UK

8-OH-DPAT hydrochloride

Whatman International Ltd., Maidstone, UK

Brandell GF/C Glassfibre filters

(+) Butaclamol hydrochloride, thioridazine hydrochloride, haloperidol base, chlorpromazine hydrochloride, spiperone hydrochloride and methiothepine maleate were generous gifts from Dr. Mark Millan, Servier, Centre de Recherches de Croissy, Croissy-sur-Seine, France.

ON1 antibody was generated against a synthetic peptide corresponding to $G_{o\alpha}$ amino acids 1-16 of the mature peptide (Mullaney and Milligan 1989).

I1C antibody was generated against a synthetic peptide corresponding to $G_{i1\alpha}$ amino acids 159-168 of the mature peptide (Green *et al.*, 1990).

2.2 Molecular Biology

2.2.1 Preparation of BL21-SI/XL1 Blue Competent Cells

An overnight culture of BL21-SI/XL1 Blue bacteria was grown in 5ml of LB broth. The following day the culture was used to inoculate 100ml of LB broth that was grown with aeration until the optical density at 550nM reached 0.48. The culture was chilled on ice for 10 minutes and spun at 1200g for 10 minutes at 4°C in sterile 2x50ml disposable plastic tubes. The supernatant was carefully removed and the cells gently resuspended in 20ml of buffer 1 (filter sterilised 0.03M KAc, 0.1M $RbCl_2$, 0.01M $CaCl_2$, 0.05M $MnCl_2$, 15% glycerol, pH 5.8 with acetic acid). The suspension was chilled on ice for 5 minutes and spun at 1200g for 10 minutes at 4°C. Following careful removal of supernatant the cell pellets were resuspended by pipetting in 2ml of buffer 2 (filter sterilised 10mM MOPS pH 6.5, 0.075M $CaCl_2$, 0.01M $RbCl_2$, 15% glycerol, pH 6.5 with concentrated HCl). Following 15 minutes on ice the samples were aliquoted and stored at –80°C until required.

2.2.2 Preparation of Antibiotic Agar Plates

LB agar (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 1.5% (w/v) agar) was autoclaved and allowed to cool before the addition of ampicillin (50μg/ml). The liquid LB agar was decanted into 90mm Petri dishes and allowed to solidify prior to storage at 4°C until required.

2.2.3 Transformation of Competent BL21-SI/XL1 Blue Cells with Plasmid DNA

To an aliquot of 50µl competent bacteria that have been allowed to thaw on ice, 10ng of DNA was added, and incubated on ice for 15 minutes. The cells are incubated at 42°C for 90 seconds and returned to ice for 2 minutes. 450µl of LB broth was added to the reaction and incubated at 37°C for 45 minutes in a shaking waterbath. 200µl of the reaction was spread onto agar plates containing ampicillin that were incubated inverted overnight at 37°C and transformed colonies selected from the following day.

2.2.4 Preparation of Plasmid DNA

Colonies transformed using XL1 Blue bacteria were picked and grown overnight in 5ml of LB broth containing ampicillin (50µg/ml). Plasmid DNA was prepared using the Promega™ Wizard Plus SV miniprep purification system as per manufacturers instructions. Preparation of larger quantities of DNA was accomplished by transferring the 5ml overnight culture into 500ml of LB broth and allowing a further overnight period of growth. The DNA was subsequently purified using the Qiagen plasmid maxi-prep kit. DNA concentration was determined by measuring the absorbance of a 1:200 dilution of the sample at 260nm where 1 absorbency unit was equivalent to 50µg/ml of double stranded DNA.

2.2.5 Plasmid DNA Digestion

Using the appropriate restriction enzymes (2-4 units) and buffer as per manufacturers instructions, 1µg of DNA was digested in a 10µl volume. Agarose gel (1% w/v) electrophoresis was used to analyse the DNA. The gel contained 40µg/ml ethidium bromide. Samples were diluted 1:3 with loading buffer and the gel run at 100 volts for 20-30 minutes in 1xTAE buffer (40mM Tris-acetate, 1mM EDTA, glacial acetic acid pH 8.0).

2.2.6 TaqMan QrtPCR

TaqMan is PCR-based assay for high-throughput quantitation of nucleic acid sequences. It combines thermal cycling and fluorescence detection enabling cycle-by-cycle detection of the increase in the amount of PCR product. Forward primers,

reverse primers and probes were designed for RGS proteins 2, 3, 4, 9 and GRK2 (see table 1) and supplied by Genosys in desiccated form. These were dissolved in TE buffer (10mM Tris HCl, 0.1mM EDTA pH 8.0). PCR conditions for the experiments were established using RGS protein cDNA. Three different primer ratios were assayed in triplicate, 50:50, 300:300 and 900:900 (nM), with the probe concentration remaining constant at 100nM and 10ng of cDNA used per reaction. Each reaction was compared to a no-template control (NTC). The assay was carried out in a 96well-plate format using a Perkin Elmer ABI Prism™ 770 Sequence Detector running Sequence Detector v1.6.3 software. Each reaction contained 2x Real Time Taqman™ Mastermix containing dNTPs (including dUTP), Hot Goldstar DNA polymerase, $MgCl_2$ (5mM final), Uracil-N-glycosylase, stabilisers and Rox Passive Reference. The thermal cycler conditions were as follows: stage 1, 2 minutes at 50°C, stage 2, 10 minutes at 95°C, and stage 3, 15 seconds at 95°C followed by 1 minute at 60°C repeated 40 times.

Table 2.1 Primers and Probes for Taqman QrtPCR

RGS Protein	Sequence
RGS2	Forward primer : GATTGGAAGACCCGTTTGAGC Reverse primer : CAGGAGAAGGCTTGATGAAAGC Probe : CTGGGAAGCCCAAAACCGGCAA
RGS3	Forward primer : CTGGTTCACAAATACGGGTTAGC Reverse primer : TCACAAGCCAACCAGAACTCC Probe : TGTTCCAAGCCTTCCTTCGCACTGA
RGS4	Forward primer : TTCATCTCAGTCCAGGCAACC Reverse primer : CTTCTGGGCCTCATCAAAGC Probe : ACCAGGGAAGAGACAAGCCGGAACAT
RGS9	Forward primer : TGATGGACTCGGAGGATGC Reverse primer : GCATTTGGGTGACATTTGAAGA Probe : TGTAAGGAAAGAGGCAGGCTGAGTTGGG
GRK2	Forward primer : CGATAAGTTCACACGGTTTTGC Reverse primer : GTATGACATGCAGACAATGAATGG Probe : CCGGAAGCGTGACACAGGCAAGAT

Following establishment of PCR conditions the assay was run using DNA from untransfected HEK293 cells. Standard curves for the RGS proteins were run at seven cDNA concentrations (10ng/µl, 1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl and 100fg/µl) alongside the HEK293 DNA sample. 18S ribosomal DNA expression was examined as a reference.

2.3 GST Fusion Protein Preparation

2.3.1 Preparation of Protein

Colonies of transformed BL21-SI bacteria were selected and grown overnight in 10ml of LB broth containing ampicillin (50μg/ml). The following morning, this culture was added to 500ml of LB broth containing ampicillin (50 μg/ml) and grown with aeration until an OD600 of 0.2 was reached. 500μl of 1M IPTG was added and the culture grown for a further 4 hours with aeration after which the sample is cooled on ice for 10 minutes. 1ml samples that were taken prior to IPTG addition, and after 2 and 4 hours incubation, were spun at 20000g to pellet the cells and 100ml loading buffer added.

The large culture was spun for 15 minutes at 6000g to pellet the cells and the supernatant discarded. The pellet was resuspended in Bugbuster™ protein extraction reagent (5ml per gram of wet pellet), 2-4μl of Benzonase® nuclease enzyme added, left on ice for 1 hour and sonicated 2x30 seconds at 60kHz using a probe sonicator. The main sample was spun at 20000g for 30 minutes and the supernatant transferred to a sterile 50ml tube containing 300μl of washed (2x1ml with sterile PBS) glutathione Sepharose® 4B gel. DTT was added to a final concentration of 5mM and the sample spun on a rotary wheel for 30 minutes at 4°C. The mixture was spun at 500g for 5 minutes, the supernatant removed and stored at –80°C, and the glutathione sepharose® 4B gel washed twice with 5ml of sterile PBS. The pellet was resuspended in 300μl of 10mM glutathione, mixed by gentle inversion and left on ice for 5 minutes. The sample was spun for 3 minutes at 500g and the supernatant removed and kept on ice. The addition of glutathione and its removal was repeated five times. A 10μl sample from each elution was removed and 90μl of SDS-PAGE loading buffer added.

2.3.2 Polyacrylamide Gel Electrophoresis

Samples were boiled at 100°C for five minutes in loading buffer (Tris 60mM, Glycerol 5%, DTT 50mM, SDS 80mM at pH 6.8) and loaded onto precast NuPAGE® Bis-Tris 4-12% gradient gels alongside full range Rainbow™ molecular weight markers. A constant 200volts was applied to the gel in MOPS SDS running buffer (3-(N-morpholino)propane sulfonic acid 50mM, Tris base 50mM, SDS

3.465mM, EDTA 1.025mM at pH 7.7) with variable AMPs until the dye front reached the end of the gel. The stacker was removed and the protein stained for using Coomassie Blue.

2.3.3 Coomassie® Protein Assay

Samples of purified RGS-GST protein were diluted 1:5, 1:10 and 1:20 to a final volume of 20µl using PBS and 480µl of Coomassie® reagent added. The mixture was vortexed briefly and triplicate 100µl aliquots added to a 96 well plate and the OD at 595nm read. Calculation of protein concentration was by comparison to a BSA standard curve.

2.4 Cell Culture

2.4.1 Cell Maintenance

HEK293 cells were grown in DMEM supplemented with 10% NBCS and 1% L-glutamine in a 37°C humidified 5% CO_2 atmosphere. DMEM for cells stably expressing the 5-HT_{1A} receptor and G protein fusion constructs also contained G-418 (1mg/ml). Confluent cells were passaged at 1:10 by the addition of 1ml of trypsin to detach the cells, 9mls of medium added and the cells pipetted gently to resuspend evenly.

2.4.2 LipofectAMINE Transfections

HEK293 cells were transiently transfected at approximately 80% confluency in 10cm^2 dishes. 10µg of DNA and 30µl of lipofectAMINE reagent were mixed gently with 450µl of OptiMEM and incubated for 45minutes. During this period, cell monolayers were washed with OptiMEM and medium replaced with 9.5ml of Optimem. The DNA-LipofectAMINE mix was added dropwise to the plates and returned to the incubator for 3 hours. The transfection medium was replaced with fresh DMEM and the cells analysed in the following 24-48 hours.

Stable transfection followed the same protocol as transient transfection with the exceptions, cells were transfected at 40-50% confluency, using serum free medium, and the cells were washed with serum free medium prior to addition of the

DNA-lipofectAMINE mix. Following incubation, the transfection mix is replaced with serum free medium and incubated for 48 hours. This medium is replaced with DMEM containing NBCS and L-glutamine with G-418 (1mg/ml) and changed daily until distinct colonies of cells could be observed. Colonies were picked and grown and selected following determination of receptor expression levels by single point pseudo-saturation radioligand binding.

2.5 Radioligand Binding

2.5.1 Preparation of Cell Membranes

Cells were grown to confluency in either $75cm^2$ or $150cm^2$ flasks the medium discarded and harvested by scraping using disposable cells scrapers. The cells were resuspended, washed using 2x10mls of ice cold PBS and spun for 5 minutes at 1000g in a refrigerated centrifuge. The supernatant was discarded, the pellet resuspended in 1ml of TE buffer (10mM Tris, 5mM EDTA pH 7.5 @ 4°C) and the mixture homogenised by 30 strokes of a chilled glass-on-glass Dounce homogeniser. The homogenate was spun for 6 minutes at 500g resulting in two fractions. The upper supernatant was removed and spun at 110000g for 30 minutes and the lower fraction discarded. The resultant pellet was resuspended in 300µl of TE buffer and following determination of protein concentration, diluted to 1µg/µl, aliquoted and stored at –80°C until required. Cells were routinely treated with 25ng/ml pertussis toxin.

2.5.2 Saturation Radioligand Binding

Specific radioligand binding over a range of concentrations determined receptor expression levels in membrane samples. Triplicate reaction mixtures were set up containing 2.5µg of protein, radioligand ranging in concentration from 0.02nM to 10nM, with non-specific binding being determined by the addition of 100µM 5-HT. The samples were incubated at 30°C for 60 minutes and subsequently harvested through a Brandell GF/C glassfibre filter using a Brandell cell harvester in a TE buffer (75mM Tris, 5mM EDTA pH 7.5). Filter discs were removed and soaked for at least 1 hour in 5ml of scintillant prior to counting of radioactivity on a Packard 1900TR Liquid Scintillation Analyser.

Specific binding was determined by the subtraction of non-specific counts from the total counts and the results plotted against radioligand concentration. Using the data analysis package Graphpad Prism total receptor expression (B_{max}) and the equilibrium dissociation constant (K_d) were calculated. An example of this calculation can be found in the appendix (Section 9.1).

2.5.3 Competition Radioligand Binding

Triplicate reaction mixtures were set up containing 2.5µg of protein, radioligand at a concentration equivalent to its K_d for the receptor, and a range of concentrations of competing "cold" ligand usually from 1nM to 100µM. Non specific binding was determined by the addition of 5-HT (100µM). Samples were incubated for either 1 hour for [^{3}H]-WAY100635 or 30 minutes for [^{3}H]-8-OH-DPAT or [^{3}H]-MPPF at 30°C prior to filtration as in the saturation binding assay. Data were analysed using Graphpad Prism, plotted as % of radioligand binding against log "cold" ligand concentration and an EC_{50} value determined using nonlinear regression. The equilibrium dissociation constant for the binding of the competing "cold" drug (K_i) was calculated using the Cheng-Prusoff equation (Cheng and Prusoff 1973). See appendix for an example of the calculation (Section 9.2).

2.5.4 Prevention Bindings

Triplicate reaction mixtures were set up containing 2.5µg of protein, radioligand at a concentration equivalent to its K_d for the receptor, and a range of concentrations of either GDP from 1nM to 100µM, or suramin from 0.1nM to 10µM. Non specific binding was determined by the addition of 5-HT (100µM). Samples were incubated for 30 minutes with either [^{3}H]-8-OH-DPAT or [^{3}H]-MPPF at 30°C prior to filtration as in the saturation binding assay. The assay buffer contained 20mM HEPES and 10mM $MgCl_2$. Data were analysed using Graphpad Prism, plotted as % of radioligand binding against log GDP/suramin concentration and an EC_{50} value determined using nonlinear regression.

2.6 Intact Cell Adenylyl Cyclase Assay

Intact cell cyclase assays were performed as described by Wong (1994) and Merkouris *et al.* (1997). Cells were split into 24 well plates and incubated in medium containing [^{3}H]-adenine at 0.5μCi/well for 16 hours prior to assay. Inhibition of forskolin (50μM) stimulated cAMP accumulation by 5-HT was measured over a 30 minute period at 37°C in the presence of the non-selective phosphodiesterase inhibitor IBMX (1mM). The reaction was terminated by the removal of the incubation medium, the addition of stop solution (5% (w/v) TCA, 1mM ATP, 1mM cAMP) and the transfer onto ice for a further 30 minutes. Cells were scraped off the plate, transferred to eppendorf tubes and spun at 20000g for 2 minutes. The [^{3}H]-cAMP in the supernatant was separated from the [^{3}H]-adenine and [^{3}H]-ATP by a two step column method (Taussig *et al.*, 1994) and counted on a Packard 1900TR Liquid Scintillation Analyser. [^{3}H]-cAMP (dpm) was expressed as a percentage of total [^{3}H] and each 5-HT treatment subsequently expressed as a percentage of forskolin stimulated [^{3}H]-cAMP production. These percentage stimulation data were plotted against 5-HT concentration and EC_{50} values estimated using nonlinear regression.

2.7 High affinity GTPase Assay

The high affinity GTPase assays were performed as in Hoffmann *et al.* (2001). Each reaction was performed in triplicate using 2.5μg of protein in a 100μl total volume over a 20 minute incubation period at 37°C in 96 well deep well plates and was terminated by the addition of 900μl of 5% (w/v) activated charcoal in 10mM orthophosphoric acid. The reaction mixture volume of 100μl contained 20mM creatine phosphate, 0.1u/μl creatine kinase, 0.2mM App[NH]p, 2mM ATP, 2mM ouabain, 200mM NaCl, 10mM $MgCl_2$, 4mM DTT, 0.2mM EDTA, 80mM Tris/HCl and [^{32}P]-GTP for 50,000cpm per reaction. Following centrifugation at 2800g for 10 minutes at 4°C a 300μl sample of supernatant was counted using a Packard Topcount NXT™ microplate scintillation counter.

High affinity GTPase activity was determined over a range of GTP concentrations (25nM-3000nM) to allow calculation of V_{max} for GTP hydrolysis and the K_m for GTP. This was measured in the absence and presence of 5-HT (100μM)

and a range of RGS protein concentrations (1nM-1μM (RGS16) or 5μM (RGS1)). The data were analysed and plotted using Graphpad Prism™ as V (pmol/mg/min) against [GTP] using a nonlinear regression one site binding hyperbola and also as V (pmol/mg/min) against V (pmol/mg/min)/S (substrate concentration) using linear regression. An example of these calculations can be found in the appendix.

High affinity GTPase activity over a range of concentrations for various ligands was also measured in the presence and absence of RGS1 (1μM) at a single GTP concentration (1μM final). The effects of the ligands were assessed by plotting GTPase activity (% of basal) against ligand concentration using Graphpad Prism and an EC_{50} value for each calculated. An example of these calculations can be found in the appendix (Section 9.3).

2.8 Western Blot Analysis

Following sample separation as in section 2.1.2, the gel was immersed in transfer buffer (bicine 25mM, Bis-Tris 25mM, EDTA 1.025mM, chlorobutanol 0.05mM at pH 7.2) and the protein was transferred onto nitrocellulose by application of a 30volt constant current for 1 hour. The nitrocellulose was blocked in 5% skimmed milk overnight and washed 5 times with PBS/0.1% Tween over a 30 minute period. Incubation with the primary antibody was in 1% skimmed milk prepared in PBS/0.1% Tween was for 1 hour, followed by 5 washed in PBS/0.1% Tween over a 30 minute period. Secondary antibody was prepared in 1% skimmed milk- PBS/0.1% Tween for 1 hour, again followed by 5 washes over 30 minutes with PBS/0.1% Tween. The nitrocellulose was incubated with a 50:50 (v/v) mixture of ECL reagents for 2 minutes prior to exposure to and development of X-ray film.

2.9 Statistical Analysis

Statistical analysis was calculated using Graphpad Prism™. The T-test calculations in chapters 1,2 and 4 comparing the results from the seven different stable cell lines may have produced falsely significant results. Comparisons should have been made using analysis of varience (ANOVA). Analysis of varience was used to examine the results in chapter 5. The GTPase assay 5-HT concentration response curves were analysed using ANOVA and the Newman-Keuls multiple comparison

post-test. The K_m and V_{max} results from the RGS protein concentration response curves were analysed using ANOVA and the Dunnetts post-test.

Unless otherwise stated in the results, data are mean +/- SEM from three similar experiments with data consisting of triplicate determinations.

Chapter 3

Intact Cell Adenylyl Cyclase Assays

3.1 Introduction

The wide range of physiological effects that 5-hydroxytryptamine has been indicated in are mediated by a large family of receptors consisting of at least 14 subtypes (Hoyer *et al.*, 1994). All of these receptors with the exception of the 5-HT_3 receptor, a ligand gated ion channel, are members of the superfamily of G protein-coupled receptors and can be characterised structurally by their seven membrane spanning domains with an extracellular N-terminus and intracellular C-terminus.

The 5-HT_{1A} receptor (Pedigo *et al.*, 1981) is one of the most highly studied members of this family and can be characterised by its uniquely high affinity for the agonist 8-OH-DPAT (Gozlan *et al.*, 1983; Hjorth *et al.*, 1982) and the antagonist WAY100635 (Fletcher *et al.*, 1993). The 5-HT_{1A} receptor is expressed in neurons, both pre- and post-synaptically, where it signals *via* activation of members of the pertussis toxin-sensitive family of G_i-like G proteins to inhibit adenylyl cyclase (De Vivo and Maayani, 1986; Weiss *et al.*, 1986) and activate inwardly rectifying K^+ channels (Andrade and Nicoll, 1986; Colino and Halliwell, 1987; Zgombick *et al.*, 1989). It is present on serotonergic neurons in raphe nuclei of the brain stem (Hammon, 1997) where it has a role in the control of the anxiety state of a subject functioning as a classical "autoreceptor" with 5-HT release activating the presynaptic 5-HT_{1A} receptor and inhibiting further 5-HT release. Partial 5-HT_{1A} agonists, such as buspirone and gepirone, are effective as anxiolytic (anti-anxiety) agents (Tunnicliff, 1991; Barret, and Vanover, 1993) and antagonists enhance the antidepressant effects of selective serotonin uptake inhibitors (S.S.R.I.s) such as fluoxetine (Prozac) (Artigas *et al.*, 1994; Artigas *et al.*, 1996).

Receptor-G protein fusions, where the C-terminal tail of the GPCR is linked directly to the N-terminal tail of the G protein, have been constructed to study in detail GPCR-G protein interaction (Seifert *et al.*, 1999; Milligan, 2000). The 5-HT_{1A} receptor has previously been fused to wild type (Cys^{351}) and pertussis toxin - resistant (Gly^{351} and Ile^{351}) forms of $G_{i1\alpha}$ (Kellett *et al.*, 1999). In a membrane high affinity GTPase assay a maximal concentration of 5-HT produced a large increase in GTPase activity. A level of pertussis toxin sufficient to ADP ribosylate all wild type $G_{i\alpha}$ completely inhibited 5-HT stimulation in membranes expressing wild type 5-$HT_{1A}/G_{i1\alpha}$, but had no effect on membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}$Gly/Ile mutants. This demonstrates a lack of coupling to the endogenous pool of G_i of the

fusion proteins. Pertussis toxin treatment reduced the basal GTPase activity of the 5-HT_{1A}-WT $G_{i1\alpha}$ fusion but had no effect on basal GTPase activity of the mutant receptor-G protein fusions, a result that has been previously been taken to indicate a lack of constitutive activity (Samama *et al.*, 1993; Lefkowitz *et al.*, 1993). However, in support of previous reports of inverse agonist activity, the ligand spiperone produced a decrease in basal (unstimulated) GTPase activity in membranes expressing 5-$HT_{1A}/G_{i1\alpha}$ and 5-$HT_{1A}/G_{i1\alpha}C^{351}$Ile fusions, but not in those expressing 5-$HT_{1A}/G_{i1\alpha}C^{351}$Gly, indicating that the Gly^{351} mutation did not allow constitutive activity. Pertussis toxin did not attenuate the effects of spiperone at the 5-$HT_{1A}/G_{i1\alpha}C^{351}$I fusion protein, a result that might indicate that the endogenous pool of G_i was not involved. Pertussis toxin treatment did attenuate the effects of spiperone for the 5-$HT_{1A}/G_{i1\alpha}$ protein.

5-HT mediated inhibition of adenylyl cyclase activation by forskolin was seen in HEK293 stable cell lines expressing each construct. Pertussis toxin abolished the inhibition mediated by the WT 5-$HT_{1A}/G_{i1\alpha}$ fusion but had no effect on cells expressing the Gly^{351} and Ile^{351} mutant fusions. 50% inhibition of cAMP production occurred at a concentration of 5-HT approximately a molar log order lower with the Ile^{351} mutant than the Gly^{351} mutant. The EC_{50} of the WT Cys^{351} was between the two.

It was decided to further characterise the 5-HT_{1A}-$G_{i1\alpha}$ fusion proteins in terms of constitutive activity and inverse agonism. With many reports indicating measurable differences in coupling between G_i and G_o α subunits (Clawges *et al.*, 1997; Bertin *et al.*, 1992; Garnovskaya *et al.*, 1997; Raymond *et al.*, 1993), parallel studies investigating coupling to the G_o class of G protein were undertaken.

3.2 Results

Prior to this project's initiation, along with the WT 5-HT_{1A} receptor, a number of 5-HT_{1A} receptor-G_α protein fusions proteins had been made. These consisted of the entire length of the 5-HT_{1A} receptor fused at the end of the C terminal tail to the N terminal tail of the G_α subunit. A schematic representation of the 5-HT_{1A} GPCR fused to the $G_{i1\alpha}$ G protein is shown in Figure 3.1. It has been previously demonstrated that the GPCR-G protein fusion did not prevent coupling to second messenger systems but may effect agonist potency (Kellett *et al.*, 1999).

G proteins of the inhibitory G_i class undergo ADP ribosylation at Cys^{351} on the G_α subunit, a reaction catalysed by pertussis toxin (Milligan, 1988). The modification prevents receptor-mediated exchange of GTP for GDP, with the G_α subunit remaining in the GDP bound inactive conformation thus preventing activation of second messenger systems. Mutation of Cys^{351} to, in this case, either Ile^{351} or Gly^{351} creates a pertussis toxin-resistant G_α subunit allowing prior pertussis toxin treatment of cells transfected with such constructs to inactivate the endogenous pool of G_i. The pharmacological advantages of this technique are vast. Previously it was not possible to quantitate the exact levels of G proteins being activated in a cell system. Now, as [^{3}H]-ligand-binding techniques allow exact quantitation of receptor levels, the 1:1 stoichiometry of the receptor to pertussis toxin-resistant G protein means that exact measurements of G protein activation can be made.

In this case both WT and mutant $G_{i1\alpha}$ and $G_{o1\alpha}$ were utilised. cDNA encoding the 5-HT_{1A} receptor with a *BamH1* site at the C terminus and encoding the appropriate $G_{i1\alpha}$ or $G_{o1\alpha}$ protein with a *BamH1* site at the N terminus were digested and ligated into pcDNA3 (Figure 3.2). These constructs were used to set up stable cell lines in HEK293 cells. In the case of the 5-HT_{1A} receptor and the case of 5-$HT_{1A}/G_{i1\alpha}$, 5-$HT_{1A}/G_{i1\alpha}C^{351}$G/I, and the 5-$HT_{1A}/G_{o1\alpha}C^{351}$G fusions, stable cell lines expressing the constructs at high levels were already available (Table 3.1). Stable cell lines were consequently created expressing the 5-$HT_{1A}/G_{o1\alpha}$ receptor and the 5-$HT_{1A}/G_{o1\alpha}C^{351}$I fusions.

For each construct 48 colonies were selected, membranes prepared and relative expression levels assayed using single point pseudo-saturation binding assays. As can be seen from Figures 3.3 and 3.4, the majority of clones expressed the constructs at sub pmol/mg levels. To allow meaningful comparison of the stable cell

lines already available and those being generated, clones with similar expression levels were chosen. Two clones of each construct were selected and full saturation binding carried out to accurately determine expression levels. For the 5-HT_{1A}/$G_{o1\alpha}$ construct these were termed Go12 and Go27, and for the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ construct these were termed GoI39 and GoI48. Figure 3.5 shows such a curve for GoI48 where [^{3}H]-MPPF, a highly selective 5-HT_{1A} antagonist (Kung *et al.*, 1996), was utilised. The data analysis package Graphpad Prism™ was used to estimate the B_{max}. This revealed expression levels of 5-6pmol/mg of protein for the Go27 and GoI48 constructs and lower expression levels for Go12 and GoI39 (Table 3.2). It was decided that the clones Go27 and GoI48 would be used in future for stable cell line work on these constructs.

A second method used to confirm expression of the 5-HT_{1A} receptor-G protein fusions was Western blotting. Although an antibody was not available for the 5-HT_{1A} receptor there were antibodies specific for the G_o and G_i subunits. Membrane samples for each of the seven stable cell lines were run on two separate gels. The G_i protein specific antibody identified the endogenous pool of G_i at approximately 40kDa as well as a band at approximately 85-90kDa that represents the receptor-G_i protein fusion proteins (Figure 3.6, panel A). The G_o protein fusion proteins did not produce a signal.

The second gel was probed using an antibody specific to $G_{o\alpha}$(ON1) in the N terminal region. The G_o fusion proteins generated a signal at approximately 85-90kDa, but the G_i fusion proteins did not (Figure 3.6, panel B). Rat brain was run as a positive control on both gels.

After establishing expression of the constructs, the next step was to demonstrate functional activity. As the 5-HT_{1A} receptor is G_i-linked, its activation results in inhibition of adenylyl cyclase. This coupling has been observed in several previous studies (Banerjee *et al.*, 1993, Fargin *et al.*, 1989, Liu and Albert, 1991, Varrault *et al.*, 1992, Kellett *et al.*, 1999). In order to look at inhibition of cAMP production, cells that had been labelled with [^{3}H]-adenine for 24 hours were treated with forskolin that binds to and directly activates adenylyl cyclase. These elevated levels of cAMP production can be inhibited with the addition of 5-HT, activating the 5-HT_{1A} receptor and its G protein fusions.

A decrease in cAMP production was observed following 5-HT stimulation using all seven stable cell lines (Figures 3.7 - 9). Following pre-treatment with pertussis toxin, abolition of 5-HT inhibition of cAMP production was seen in the 5-HT_{1A}, 5-$HT_{1A}/G_{i1\alpha}$ and 5-$HT_{1A}/G_{o1\alpha}$ stable cell lines. The ADP ribosylation of Cys^{351} of endogenous and WT fused inhibitory G proteins prevents G protein activation and thus inhibition of adenylyl cyclase activity. Pertussis toxin treatment did not have a significant effect on 5-HT inhibition of cAMP production by the four ADP ribosylation resistant G protein receptor fusions. This indicates that for the mutant fusion proteins, ligand-activated signal transduction in this assay is conferred by the fused G protein only. If the 5-HT_{1A} receptor – Ile^{351} and Gly^{351} G protein fusions were coupling to the endogenous pool of inhibitory G proteins a difference between non-, and pertussis toxin-treated cells should have been apparent.

In figure 3.7, it is demonstrated that fusing the WT inhibitory G proteins to the 5-HT_{1A} receptor produces a significant increase in the EC_{50} for inhibition of adenylyl cyclase ($P < 0.05$), with an EC_{50} for the 5-HT_{1A} receptor alone being approximately ten fold lower (3.9+/-0.7nM versus 52.4+/-17.6nM and 48.0+/-16.4nM) (Table 3.3). This may suggest that the ligand stimulated wild type receptor is capable of coupling to more than one G protein, resulting in the inhibition of a larger proportion of the forskolin activated adenylyl cyclase at the same level of receptor occupancy.

Examining the results for the $G_{i1\alpha}$ fusions in isolation, in terms of signal transduction, 5-HT was most potent in the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ cell line, with an EC_{50} of approximately 2nM (Figure 3.8). In decreasing order of potency, the WT $G_{i1\alpha}$ fusion had an EC_{50} of 14nM for 5-HT, followed by the Gly^{351} mutant with an EC_{50} of approximately 60nM. The observed Hill slopes followed a similar order, with the steepest being in the order of –1.7 for the Ile^{351} mutant and the shallowest being –0.9 for the Gly^{351} mutant (Table 3.3). The WT fusion again fell between the two with a Hill slope of –1.2. These results will be discussed in detail later.

The results for the $G_{o1\alpha}$ fusions followed the same pattern, although in a less pronounced fashion (Figure 3.9). The 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion had an EC_{50} of approximately 8nM and a Hill slope of between –1.6 and –1.9 (Table 3.4). The WT fusion had an EC_{50} of 42 nM with a slope of –1.9, and finally the Gly^{351} mutant had

an EC_{50} of between 43 and 59nM with a Hill slope of between –0.9 and –1.5. Again these results will be examined in the discussion.

Comparison of the inhibition of cAMP production resulting from stimulation of the 5-$HT_{1A}/G_{i1\alpha}$ fusions and the 5-$HT_{1A}/G_{o1\alpha}$ fusions showed a general trend towards greater inhibition at a lower concentration of 5-HT for the $G_{i1\alpha}$ fusion proteins.

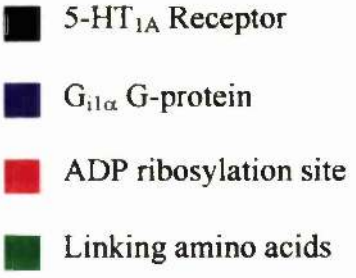
It is not clear whether inhibition of adenylyl cyclase activity by 5-HT_{1A} receptor activation of the $G_{o1\alpha}$ protein is mediated by the $G_{o\alpha}$ protein or the $G_{\beta\gamma}$ dimer. In an attempt to ascertain if $G_{\beta\gamma}$ subunits were involved the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein was co-transfected transiently into HEK293 cells with the α subunit of transducin (Figure 3.10). It was hypothesised that free $G_{\beta\gamma}$ subunits released by 5-HT activation of the fusion protein would bind to transducin α and a decrease in inhibition of adenylyl cyclase activity would be recorded if the $G_{\beta\gamma}$ dimer were involved. As not all cells would express the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ and α subunit of transducin cDNA, forskolin stimulation of adenylyl cyclase activity would mask any 5-HT_{1A} receptor-mediated effects. Adenylyl cyclase was stimulated by co-transfection and activation of the β_2-adrenergic receptor that is G_s linked. No difference was found between cells not transfected or transfeced with the α subunit of transducin cDNA but no conclusion can be drawn from this as expression was not confirmed using western blot analysis.

Another component of the intact cell adenylyl cyclase results to take note of was the tendency for an increase in cAMP production at concentrations of 5-HT greater than 1μM. This effect was most visible in the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ results and those of the pertussis toxin-treated 5-HT_{1A} receptor. As pertussis toxin had no effect on this adenylyl cyclase stimulation it could be suggested that another 5-HT receptor which is G_s linked may be present endogenously in HEK293 cells at low levels.

In order to confirm this hypothesis, a whole cell cyclase assay was carried out on un-transfected HEK293 cells. 5-HT produced an increase in basal and forskolin-stimulated cAMP production at concentrations greater than 1μM (Figures 3.11 and 3.12). These data will be interpreted fully in the Discussion.

Figure 3.1 **2D snake diagram of the human 5-HT_{1A} receptor – rat $G_{i1\alpha}$ protein fusion**

The human 5-HT_{1A} receptor was fused to both wild type and mutant inhibitory G proteins. The point of fusion was at the C terminal end of the 5-HT_{1A} receptor and the N terminal end of the G protein. Both $G_{i1\alpha}$ and $G_{o1\alpha}$ proteins were utilised and Cys^{351} mutated to Gly^{351} and Ile^{351}. Shown in the diagram is the human 5-HT_{1A} receptor joined at the C terminal to the N terminal of $G_{i1\alpha}$, using G and S amino acids as a linker. A 341 amino acid section of the $G_{i1\alpha}$ protein has been removed to allow presentation.



C
341
AA

Figure 3.2 Strategy for construction of plasmids expressing human 5-HT_{1A}/rat G_{α} fusion proteins

The 5-HT_{1A} receptor and the appropriate G protein were digested out of the pcDNA3 vector using *HindIII* and *BamHI*. Following gel purification, the appropriate cDNA segments were ligated into pcDNA3 forming the receptor-G protein fusion. The *BamHI* site produces a 6-substrate element that encodes the GS amino acid linker sequence. The stop codon from the 5-HT_{1A} cDNA and the start codon from the G_{α} protein cDNA were removed.

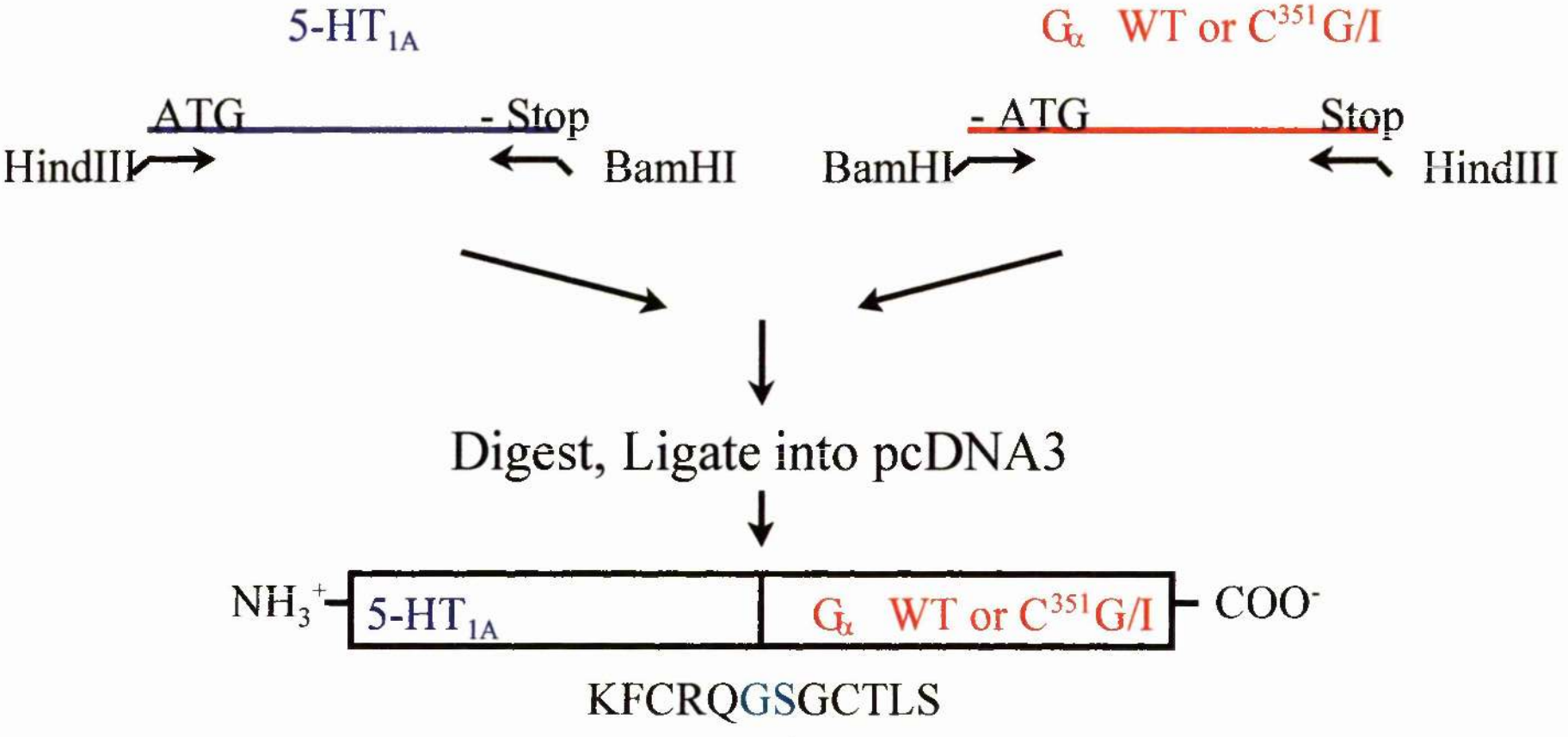

5-HT1A
Gα WT or C351G/I
ATG
- Stop
- ATG
Stop
HindIII
BamHI
BamHI
HindIII
Digest, Ligate into pcDNA3
NH3+
5-HT1A
Gα WT or C351G/I
COO-
KFCRQGSGCTLS

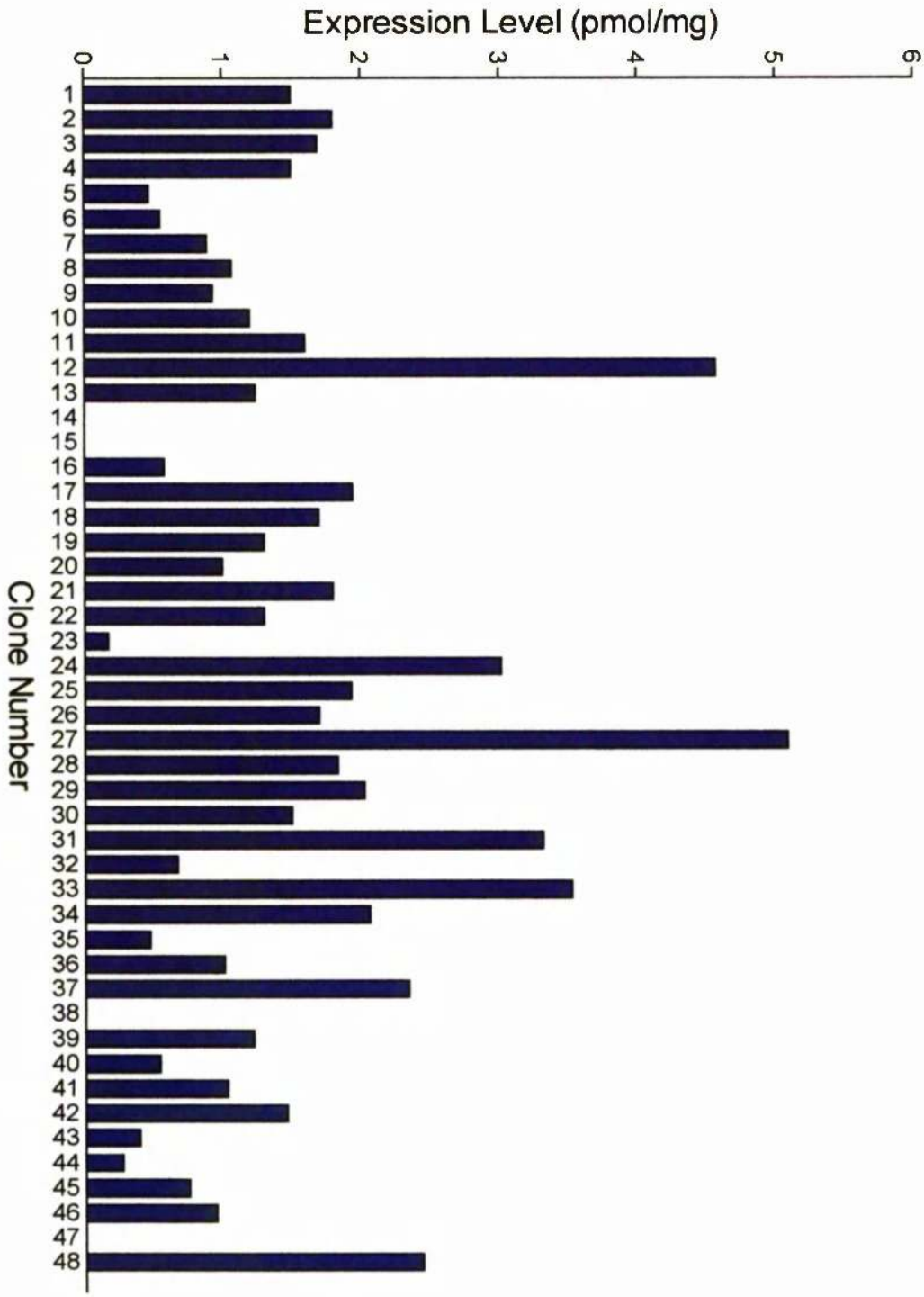
Table 3.1 Expression of the 5-HT_{1A} receptor and its G protein fusions in HEK293 stable cell lines

Saturation binding assays were used to determine B_{max} for the 5-HT_{1A} receptor and its inhibitory G protein fusions following stable expression in HEK293 cells. Membranes were prepared from harvested cells and [^{3}H]-MPPF saturation binding carried out and data analysed using Graphpad Prism as described in Methods (Section 2.5.2). Data are mean+/-SEM for three separate experiments.

Construct Expressed	B_{max} **(pmol/mg)**
5-HT_{1A}	6.3+/-0.5
5-$HT_{1A}/G_{i1\alpha}$	4.4+/-0.2
5-$HT_{1A}/G_{i1\alpha}C^{351}G$	8.6+/-0.9
5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox	9.5+/-0.5
5-$HT_{1A}/G_{i1\alpha}C^{351}I$	8.6+/-0.4
5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox	9.4+/-0.7
5-$HT_{1A}/G_{o1\alpha}C^{351}G$	7.4+/-0.2
5-$HT_{1A}/G_{o1\alpha}C^{351}G$ ptox	8.6+/-0.2

Figure 3.3 **Single point pseudo saturation analysis of [^{3}H]-MPPF binding to membranes expressing 5-$HT_{1A}/G_{o1\alpha}$ fusion proteins**

HEK293 colonies growing in DMEM containing 0.5mg/ml genetecin were picked, split into two 60mm dishes and grown to confluency. One dish was harvested, membranes prepared and single point pseudo saturation binding carried out using the 5-HT_{1A} receptor selective antagonist [^{3}H]-MPPF. Non-specific binding was determined using 10μM 5-HT. An example of the calculation of expression in pmol/mg can be found in the appendix (Section 9.1). Data are from a single experiment.


Expression Level (pmol/mg)
0
1
2
3
4
5
6
Clone Number
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
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19
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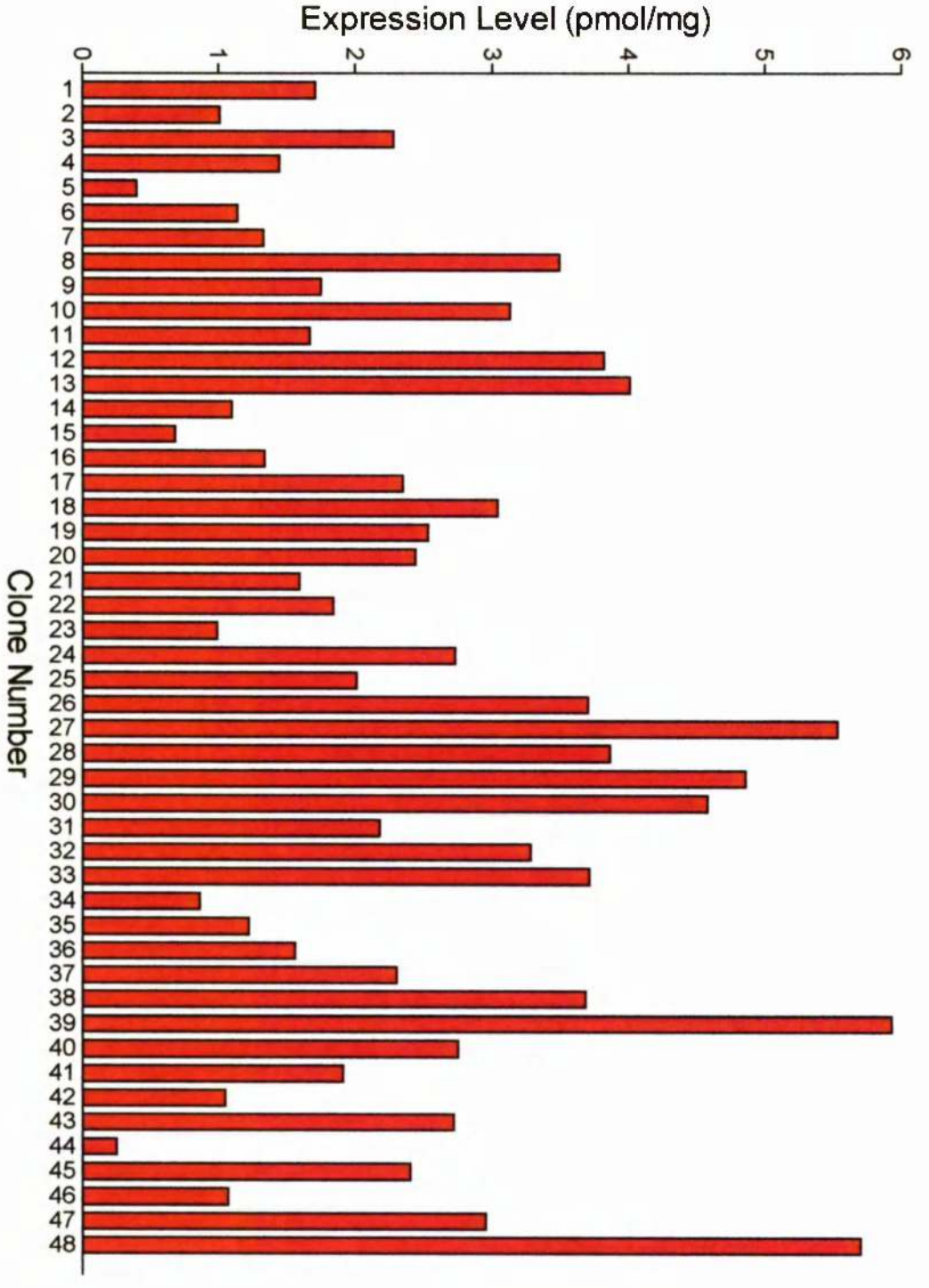
Figure 3.4 **Single point pseudo saturation analysis of [^{3}H]-MPPF binding to membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein**

HEK293 colonies growing in DMEM containing 0.5mg/ml genetecin were picked, split into two 60mm dishes and grown to confluency. One dish was harvested, membranes prepared and single point pseudo saturation binding carried out using the 5-HT_{1A} receptor selective antagonist [^{3}H]-MPPF. Non-specific binding was determined using 10μM 5-HT. An example of the calculation of expression in pmol/mg can be found in the appendix (Section 9.1). Data are from a single experiment.


Expression Level (pmol/mg)
0
1
2
3
4
5
6
Clone Number
1
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4
5
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7
8
9
10
11
12
13
14
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Figure 3.5 **Saturation analysis of [^{3}H]-MPPF binding to the 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I stable clone GoI48.**

Membranes were prepared from the GoI48 clone stably expressing the 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I fusion and a saturation-binding assay carried out using the 5-HT$_{1A}$ receptor selective antagonist [^{3}H]-MPPF. Non-specific was determined using 10μM 5-HT. Using the data analysis package Graphpad Prism, nonlinear regression analysis of the data fitted a single binding site, determining total number of binding sites (B_{max}) and the equilibrium dissociation constant (K_d). Data are means of triplicate determinations and are representative of at least three similar experiments.

■ 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I

▲ 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I ptox

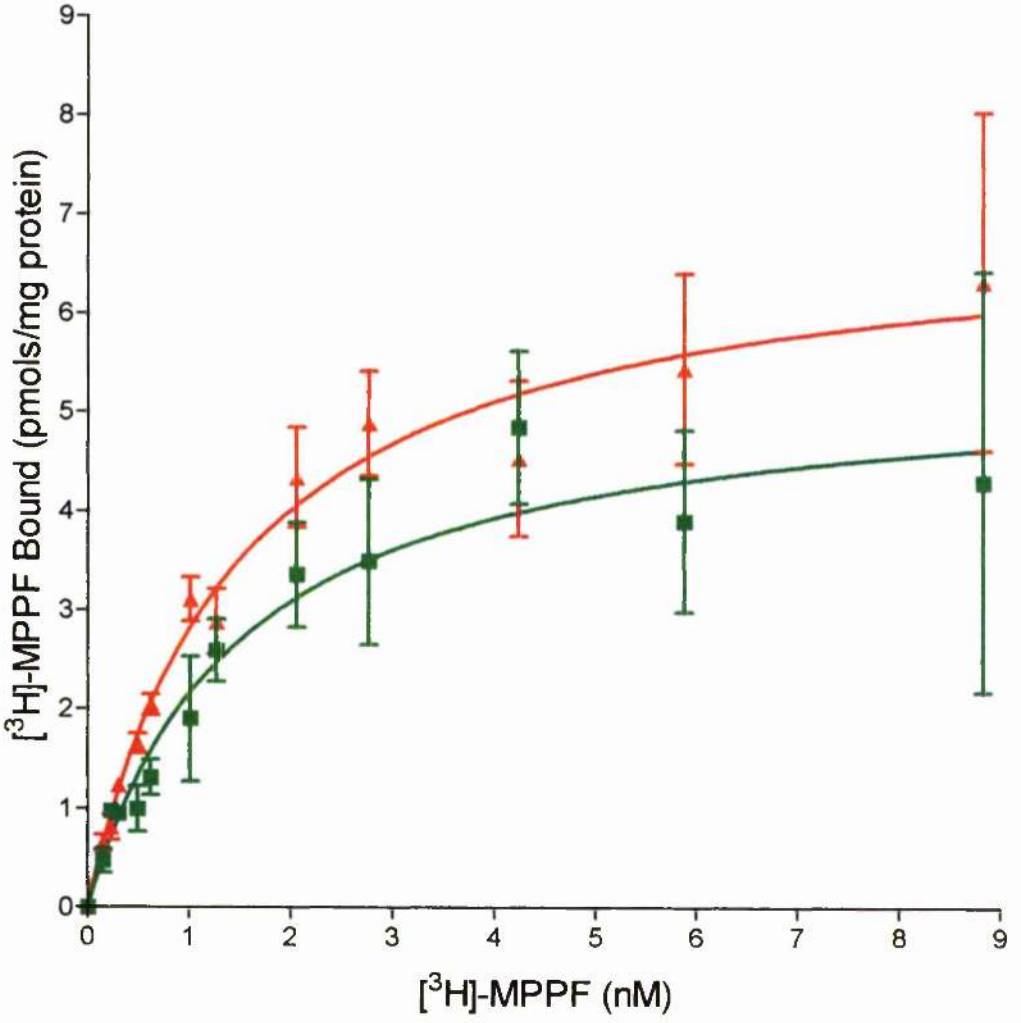

[3H]-MPPF Bound (pmols/mg protein)
[3H]-MPPF (nM)
0
1
2
3
4
5
6
7
8
9

Table 3.2 **Expression of 5-$HT_{1A}/G_{o1\alpha}$ fusion proteins as determined by [^{3}H]-MPPF saturation binding analysis.**

Following single point pseudo saturation analysis of clones expressing the 5-$HT_{1A}/G_{o1\alpha}$ fusions, two clones expressing either construct were selected, and full [^{3}H]-MPPF saturation analysis carried out on prepared membranes (see Fig. 3.5). Nonlinear regression analysis was used to determine B_{max} (pmol/mg) and the equilibriun dissociation constant (K_d) in Graphpad Prism. Data are Mean +/- SEM from three separate experiments.

Construct Expressed	**Clone**	***B_{max}*** **(pmol/mg)**	**K_d of [^{3}H]-MPPF (nM)**
5-HT_{1A}/$G_{o1\alpha}$	Go12	3.6+/-0.2	1.53+/-0.05
		3.3+/-0.3 ptox	
	Go27	4.8+/-0.5	1.62+/-0.08
		6.3+/-0.6 ptox	
5-HT_{1A}/$G_{o1\alpha}C^{351}I$	GoI39	4.6+/-0.4	1.45+/-0.10
		5.8+/-0.8 ptox	
	GoI48	5.5+/-0.6	1.50+/-0.11
		7.0+/-1.0 ptox	

Figure 3.6 **Western blot analysis of G protein expression in cells stably expressing the 5-HT_{1A} receptor and its G protein fusions**

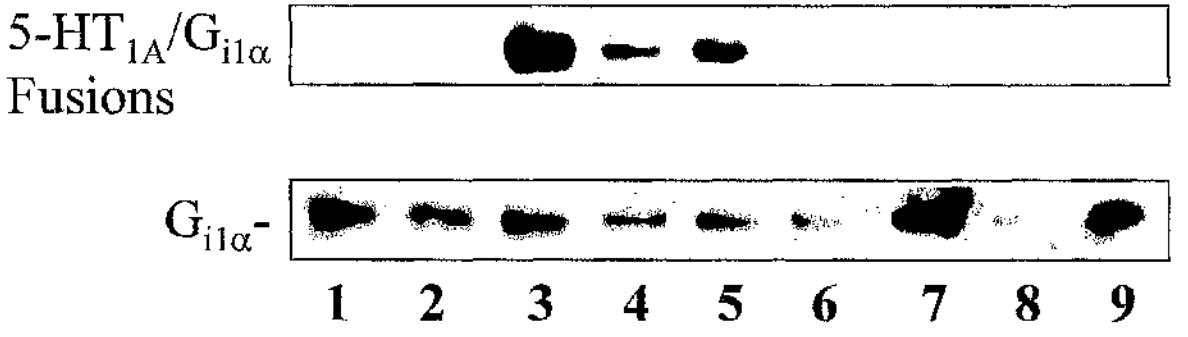
Membranes from rat brain cortex (1), and cells stably expressing the 5-HT_{1A} receptor (2), the 5-$HT_{1A}/G_{i1\alpha}$ fusion (3), the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ fusion (4), the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion (5), the 5-$HT_{1A}/G_{o1\alpha}$ fusion (6), the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion (7), the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion (8) and mock transfected HEK293 cells (9) were resolved by SDS page.

Panel A: Immunoblot detection of the 5-$HT_{1A}/G_{i1\alpha}$ fusion constructs and endogenous $G_{i1\alpha}$ with antiserum I1C (Green *et al.*, 1990), which identifies amino acids 159 to 168 of $G_{i1\alpha}$. Top, detection of the fusion proteins. Bottom, detection of endogenous $G_{i1\alpha}$.

Panel B: Immunoblot detection of the 5-$HT_{1A}/G_{o1\alpha}$ fusion constructs and endogenous $G_{o\alpha}$ with antiserum ON1, which identifies amino acids 1 to 16 of $G_{o\alpha}$. Top, detection of the fusion proteins. Bottom, detection of endogenous $G_{o1\alpha}$.

Equal amounts of protein were added for each sample. Blots are representative of three such experiments carried out on different membrane samples.

Panel A



Panel B

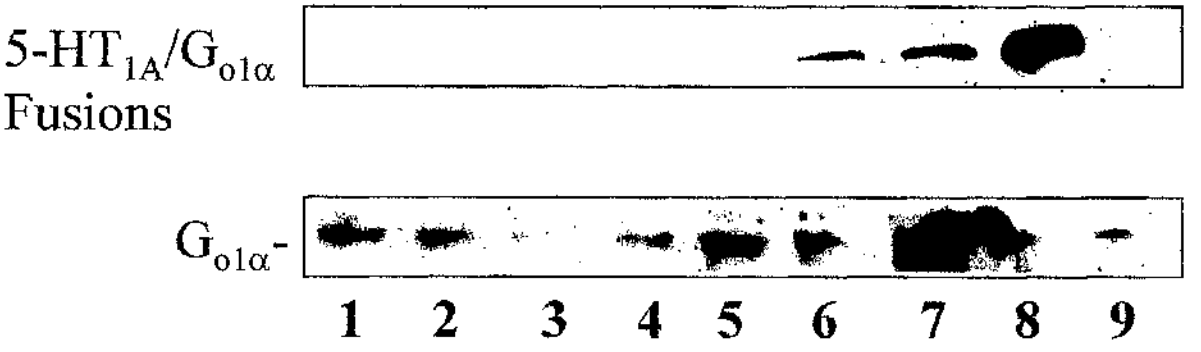


Figure 3.7 **Adenylyl cyclase regulation in intact cells expressing the 5-HT_{1A} receptor and WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusions**

Concentration dependent 5-HT inhibition of 50μM forskolin stimulated adenylyl cyclase activity in HEK293 cells stably expressing the 5-HT_{1A} receptor, or WT inhibitory G protein fusions. 100% is defined as maximal incorporation of [^{3}H]-adenine into cAMP following forskolin mediated stimulation of adenylyl cyclase. Data were fitted using nonlinear regression analysis (Graphpad Prism). Data are means of triplicate determinations and are representative of at least three similar experiments.

- 5-HT_{1A}
- 5-HT_{1A} ptox
- 5-$HT_{1A}/G_{i1\alpha}$
- 5-$HT_{1A}/G_{i1\alpha}$ ptox
- 5-$HT_{1A}/G_{o1\alpha}$
- 5-$HT_{1A}/G_{o1\alpha}$ ptox

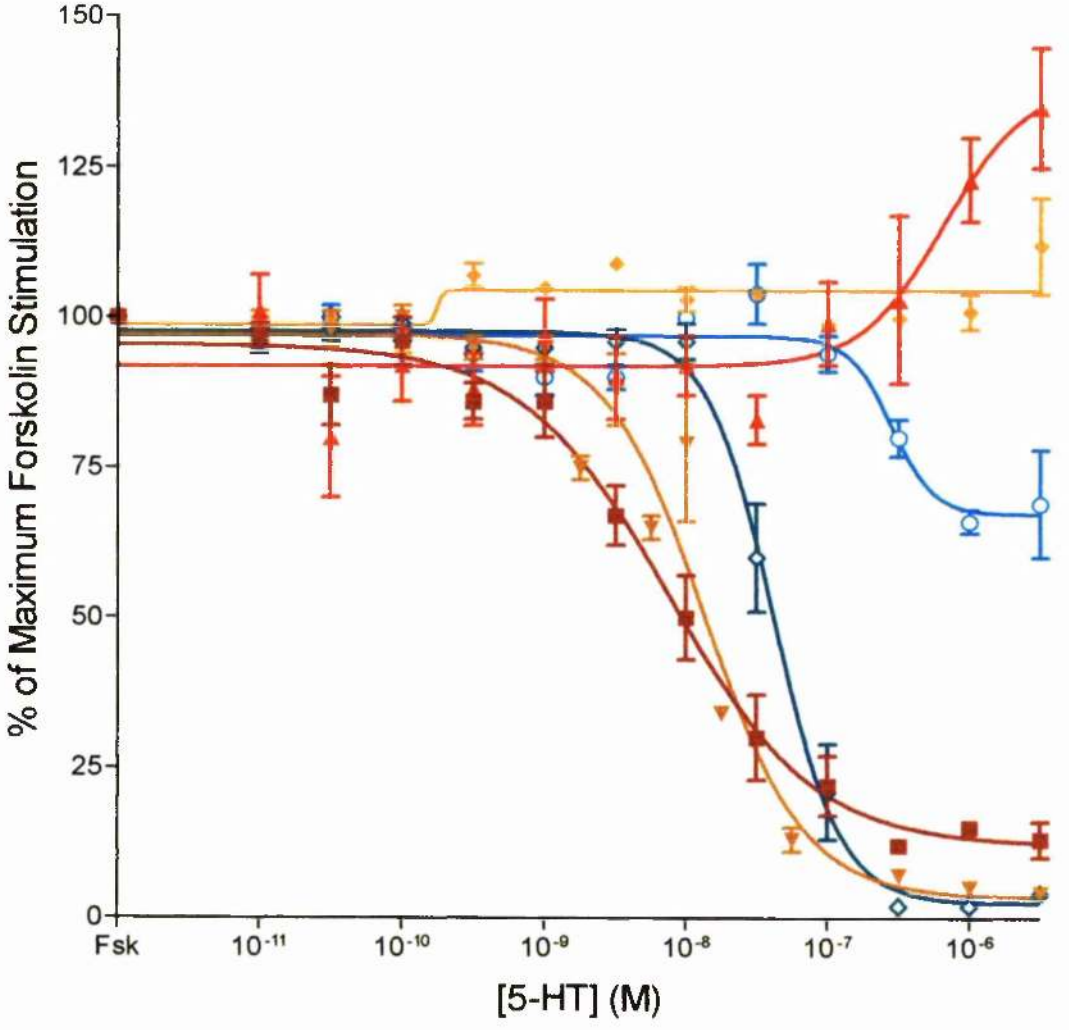

150
125
100
75
50
25
0
% of Maximum Forskolin Stimulation
Fsk
10-11
10-10
10-9
10-8
10-7
10-6
[5-HT] (M)

Figure 3.8 **Adenylyl cyclase regulation in intact cells expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}G/I$ fusions**

Concentration dependent 5-HT inhibition of 50μM forskolin stimulated adenylyl cyclase activity in HEK293 cells stably expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}G/I$ fusions. 100% is defined as maximal incorporation of [^{3}H]-adenine into cAMP following direct stimulation of adenylyl cyclase with 50μM forskolin. Data were fitted using nonlinear regression analysis (Graphpad Prism). Data are means of triplicate determinations and are representative of at least three similar experiments.

5-$HT_{1A}/G_{i1\alpha}C^{351}G$

5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox

5-$HT_{1A}/G_{i1\alpha}C^{351}I$

5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox

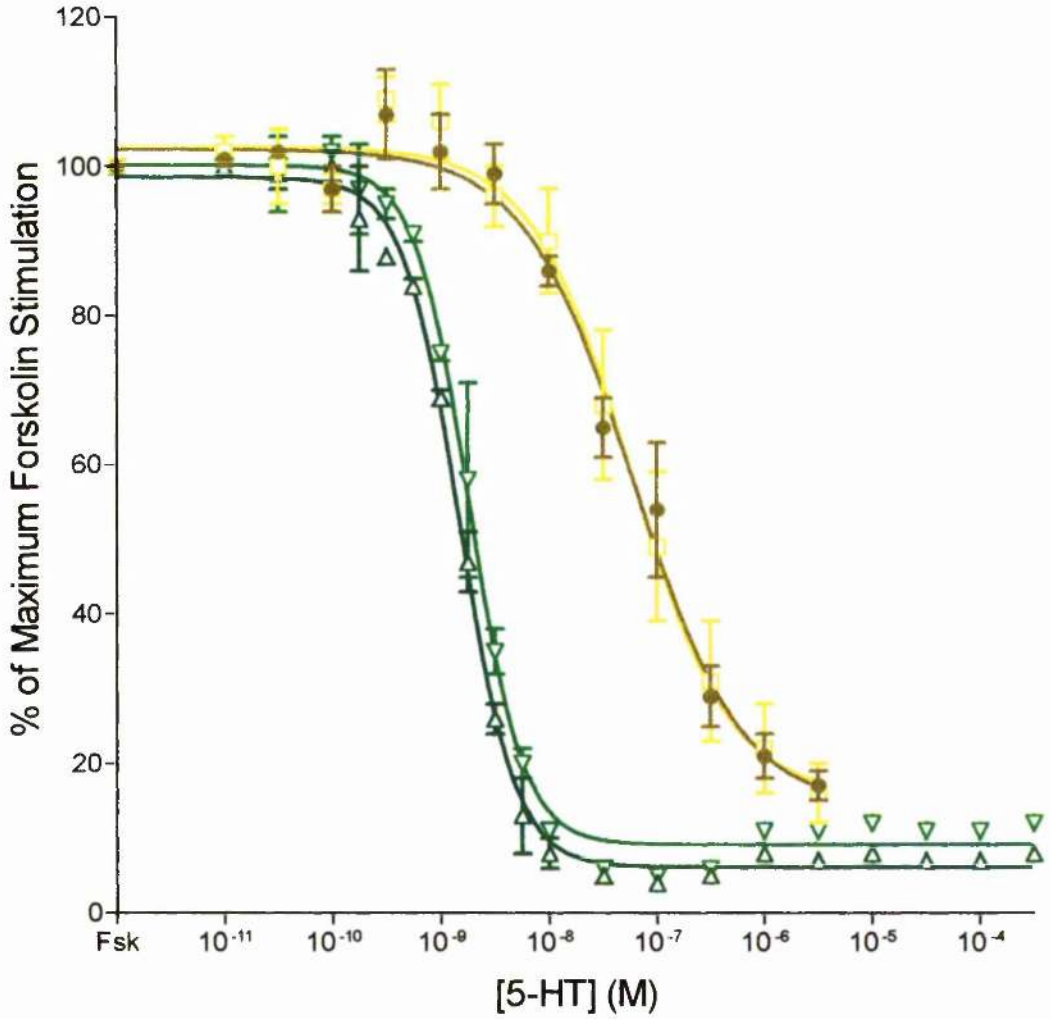

120
100
80
60
40
20
0
% of Maximum Forskolin Stimulation
Fsk
10-11
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[5-HT] (M)

Figure 3.9 **Adenylyl cyclase regulation in intact cells expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G/I$ fusions**

Concentration dependent 5-HT inhibition of 50μM forskolin stimulated adenylyl cyclase activity in HEK293 cells stably expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G/I$ fusions. 100% is defined as maximal incorporation of [^{3}H]-adenine into cAMP following direct stimulation of adenylyl cyclase with 50μM forskolin. Data were fitted using nonlinear regression analysis (Graphpad Prism). Data are means of triplicate determinations and are representative of at least three similar experiments.

□ 5-$HT_{1A}/G_{o1\alpha}C^{351}G$

△ 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ ptox

▽ 5-$HT_{1A}/G_{o1\alpha}C^{351}I$

◇ 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ ptox

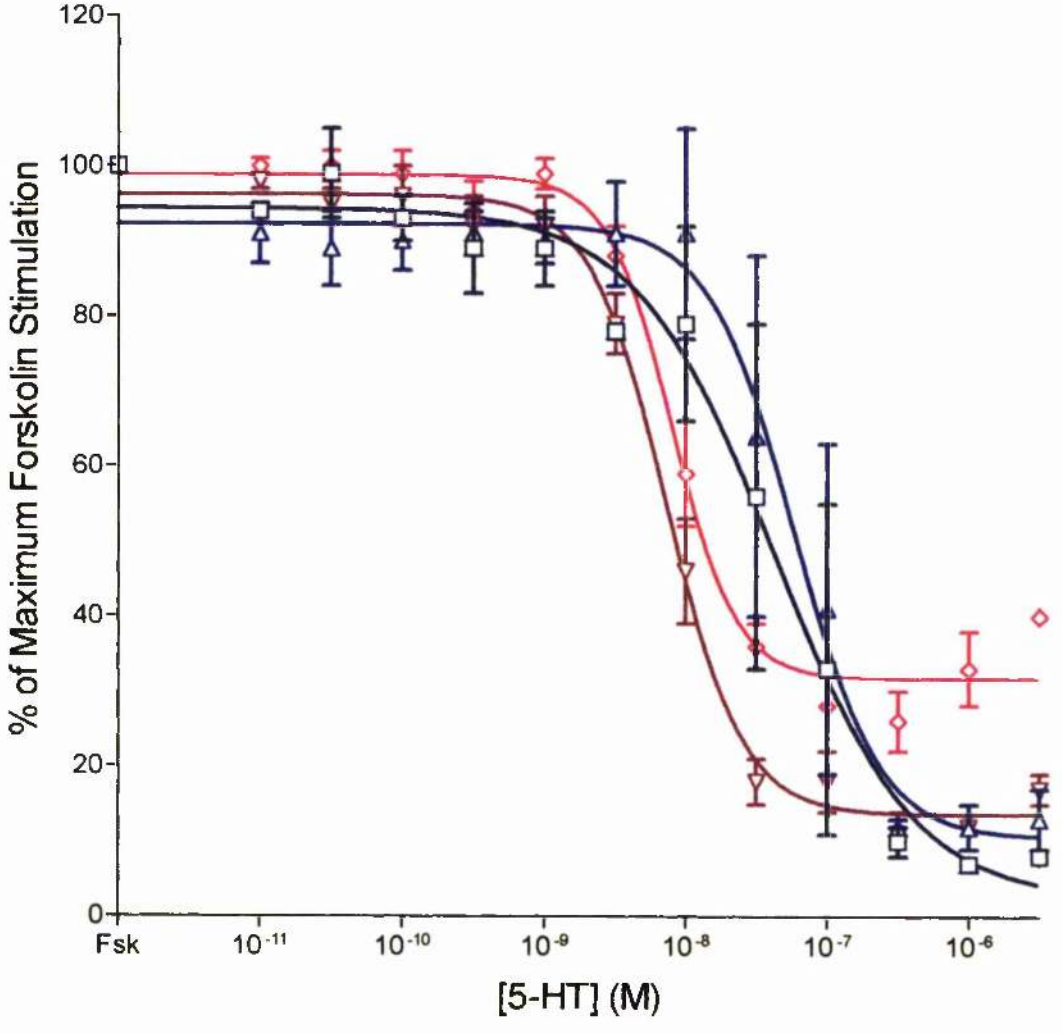

120
100
80
60
40
20
0
% of Maximum Forskolin Stimulation
Fsk
10-11
10-10
10-9
10-8
10-7
10-6
[5-HT] (M)

Table 3.3 **EC_{50} and Hill slope determinations from whole cell adenylyl cyclase assay of the 5-HT_{1A} receptor and $G_{i1\alpha}$ protein fusions**

5-Hydroxytryptamine EC_{50} values for inhibition of forskolin (50μM) stimulated adenylyl cyclase activity in cells stably expressing the 5-HT_{1A} receptor and WT and mutant $G_{i1\alpha}$ protein fusions. Data were analysed by nonlinear regression using the Graphpad Prism data analysis package. Average EC_{50} and Hill slope values following either three or four replications.

Construct Expressed	**EC_{50} (nM) Mean +/-SEM**	**Hillslope Mean +/- SEM**	**Number of Replicates**
5-HT_{1A}	3.9 +/- 0.7	-1.4 +/- 0.4	3
5-$HT_{1A}/G_{i1\alpha}$	52.4 +/- 17.6	-1.3 +/- 0.2	3
5-$HT_{1A}/G_{i1\alpha}C^{351}G$	81.9 +/- 22.8	-0.8 +/- 0.3	4
5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox	68.0 +/- 23.1	-1.1 +/- 0.1	4
5-$HT_{1A}/G_{i1\alpha}C^{351}I$	1.5 +/- 0.1	-1.7 +/- 0.03	3
5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox	1.9 +/- 0.4	-1.8 +/- 0.01	3

Table 3.4 **EC_{50} and Hill slope determinations from whole cell adenylyl cyclase assay of the 5-HT_{1A} receptor and $G_{o1\alpha}$ protein fusions**

5-Hydroxytryptamine EC_{50} values of inhibition of forskolin (50μM) stimulated adenylyl cyclase activity in cells stably expressing the 5-HT_{1A} receptor and WT and mutant $G_{o1\alpha}$ protein fusions. Data were analysed by nonlinear regression using the Graphpad Prism data analysis package. Average EC_{50} and Hill slope values following three replications.

Construct Expressed	EC_{50} (nM) Mean +/-SEM	Hillslope Mean +/- SEM	Number of Replicates
5-HT_{1A}	3.9 +/- 0.7	-1.4 +/- 0.4	3
5-$HT_{1A}/G_{o1\alpha}$	48.0 +/- 16.4	-2.3 +/- 0.3	3
5-$HT_{1A}/G_{o1\alpha}C^{351}G$	14.7 +/- 8.0	-1.3 +/- 0.3	3
5-$HT_{1A}/G_{o1\alpha}C^{351}G$ ptox	19.7 +/- 10.7	-1.9 +/- 0.5	3
5-$HT_{1A}/G_{o1\alpha}C^{351}I$	7.7 +/- 1.3	-1.4 +/- 0.1	3
5-$HT_{1A}/G_{o1\alpha}C^{351}I$ ptox	9.0 +/- 3.1	-1.6 +/- 0.2	3

Figure 3.10 **Inhibition of isoprenaline stimulated adenylyl cyclase activity by agonist activation of the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein in the absence and presence of transducin α**

The 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein was transiently transfected into HEK293 cells with the β_2 adrenoceptor in the presence and absence of transducin α transfection. The ability of 5-HT activation of the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein to inhibit β_2 activation of adenylyl cyclase activity following isoprenaline stimulation was then studied. Data points are mean values of triplicate replications and are representative of at least three individual experiments.

5-$HT_{1A}/G_{o1\alpha}C^{351}G$

5-$HT_{1A}/G_{o1\alpha}C^{351}G$ + Transducin α

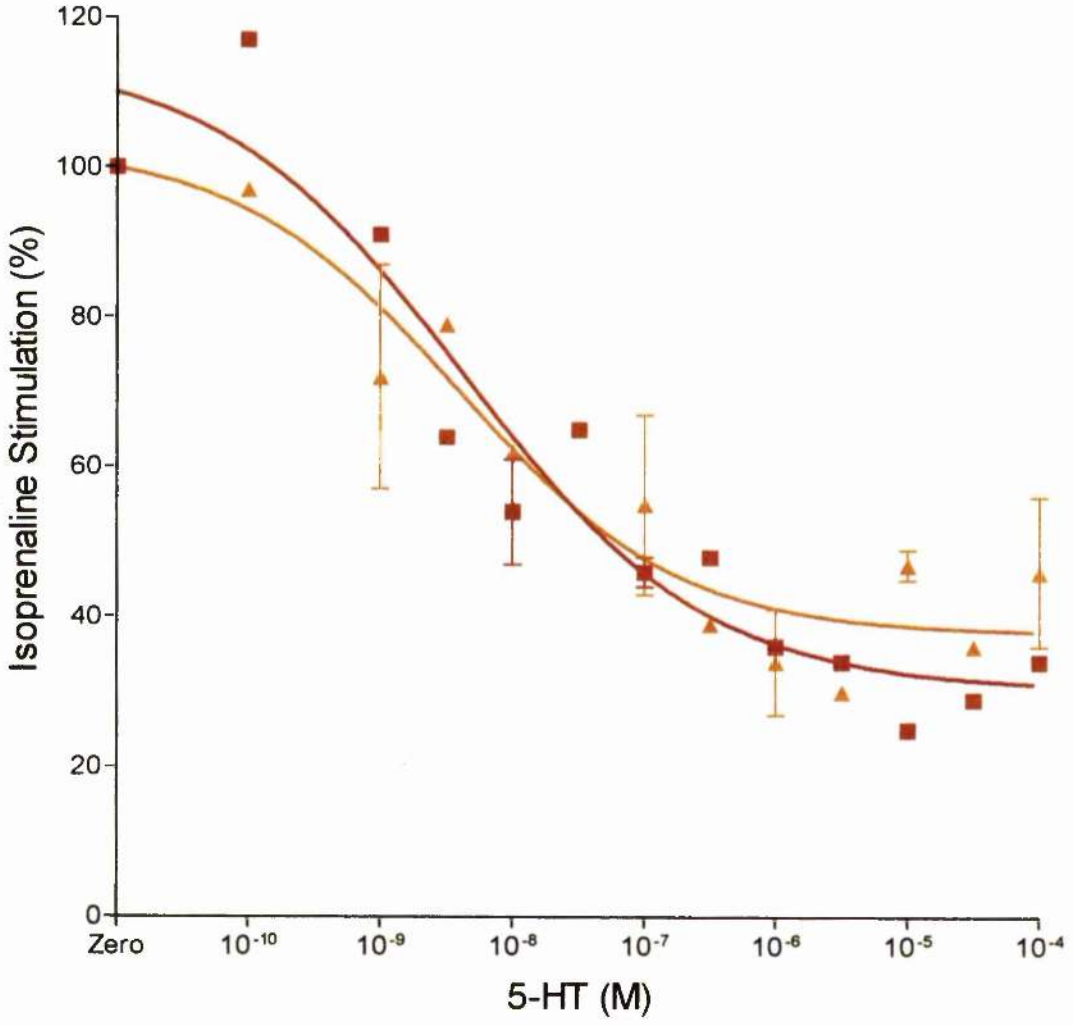

120
100
80
60
40
20
0
Isoprenaline Stimulation (%)
Zero
10-10
10-9
10-8
10-7
10-6
10-5
10-4
5-HT (M)

Figure 3.11 Adenylyl cyclase regulation in non-transfected intact HEK293 cells

Concentration dependent 5-HT stimulation of adenylyl cyclase activity in non-transfected HEK293 cells. Data are expressed as the % of total [^{3}H]-Adenine incorporated into cAMP and was fitted using nonlinear regression analysis (Graphpad Prism). Data are means of triplicate determinations and are representative of at least three similar experiments.

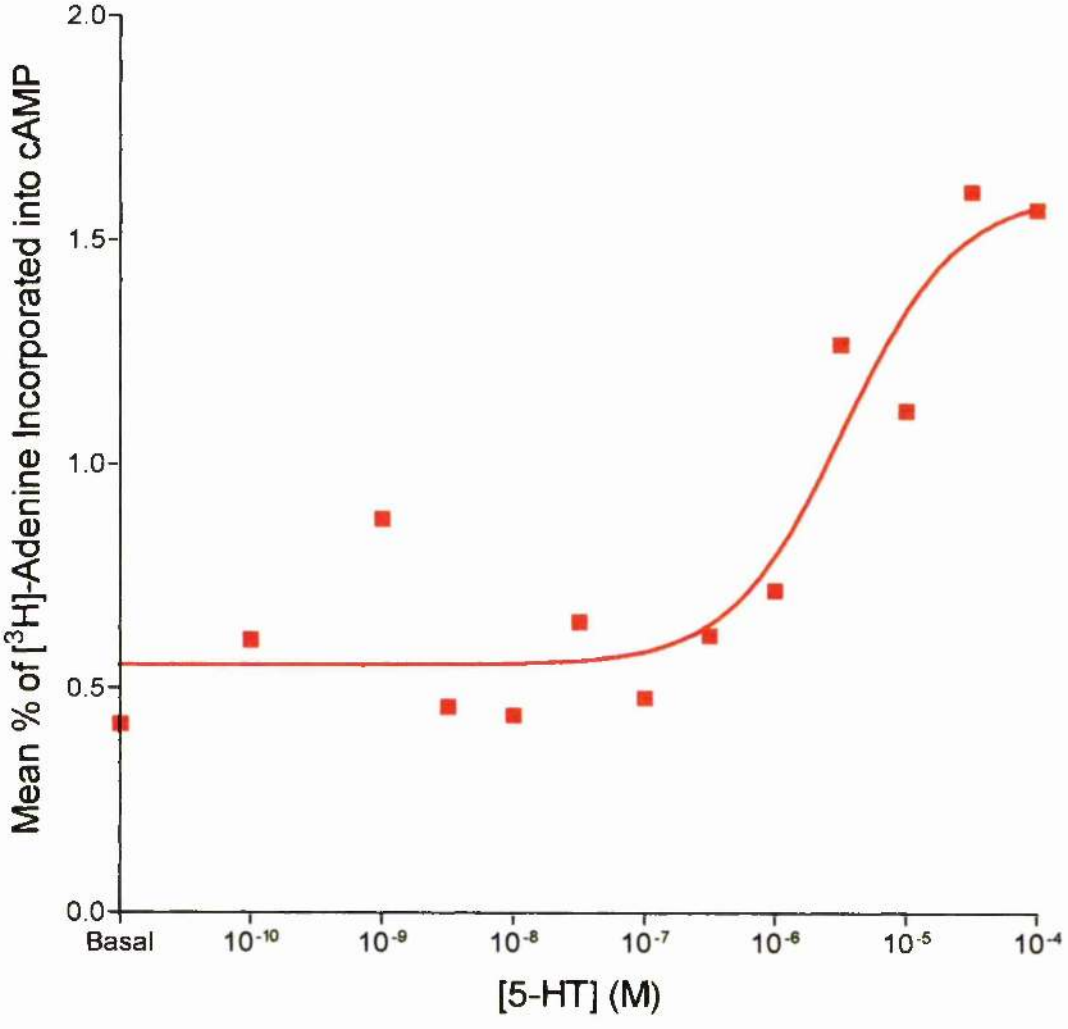
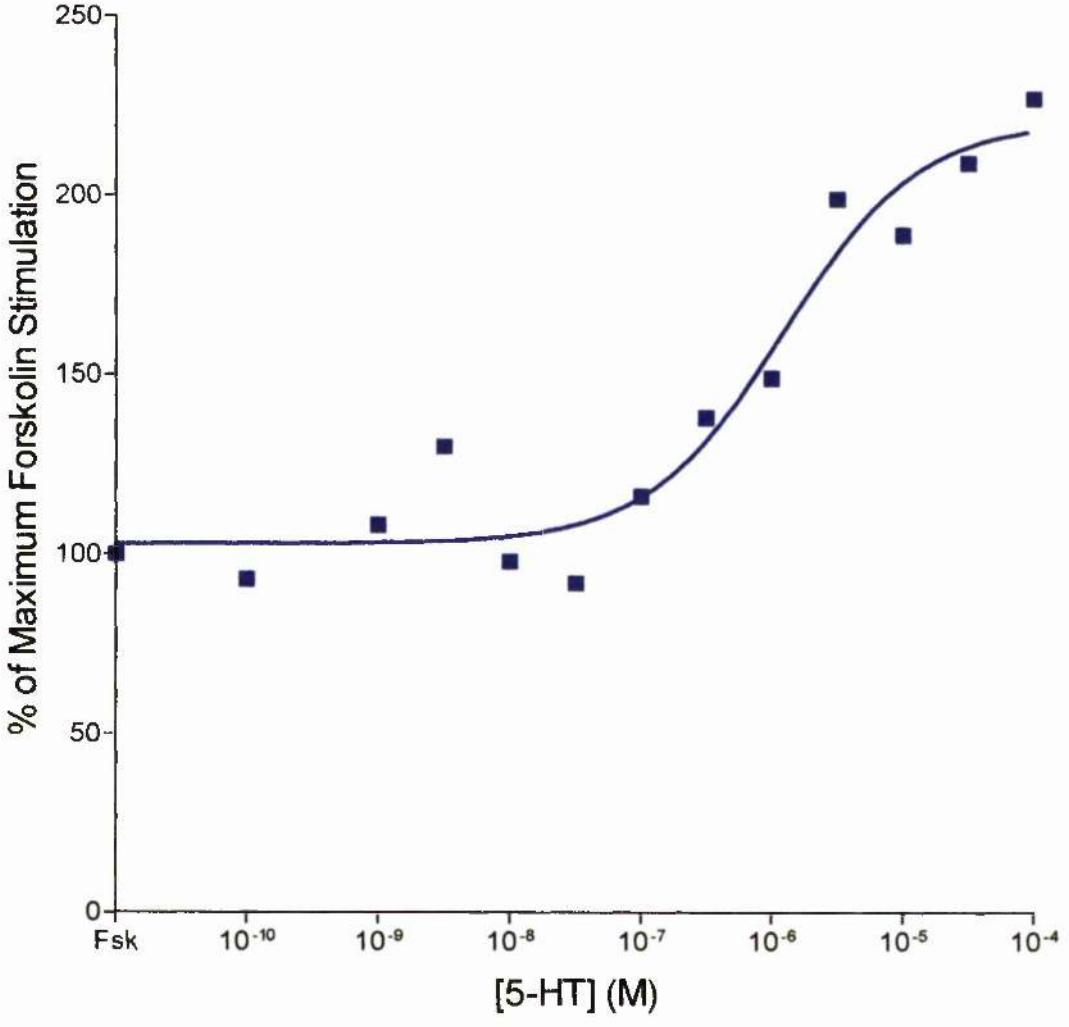

Mean % of [3H]-Adenine Incorporated into cAMP
2.0
1.5
1.0
0.5
0.0
Basal
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[5-HT] (M)

Figure 3.12 Adenylyl cyclase regulation in non-transfected intact HEK293 cells

Concentration dependent 5-HT stimulation of adenylyl cyclase activity in non-transfected HEK293 cells following 50μM forskolin activation. 100% is defined as maximal incorporation of [^{3}H]-adenine into cAMP following forskolin treatment and data was fitted using nonlinear regression analysis (Graphpad Prism). Data are means of triplicate determinations and are representative of at least three similar experiments.


250
200
150
100
50
0
% of Maximum Forskolin Stimulation
Fsk
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[5-HT] (M)

3.3 Discussion

The 5-HT_{1A} receptor (Pedigo *et al.*, 1981), which has been studied extensively, has been indicated as having an important role in determining the human emotional state. It is present both pre- and post-synaptically on serotonergic neurons in raphe nuclei of the brain stem (Hammon, 1997). These cells are responsible for the synthesis of the majority of the 5-HT within the brain and project throughout the C.N.S. The partial 5-HT_{1A} agonists, buspirone and gepirone, are effective anxiolytics (Tunnicliff, 1991; Barret, and Vanover, 1993), and the effects of the S.S.R.I., Prozac (fluoxetine), are enhanced and accelerated by co-administration of 5-HT_{1A} antagonists (Artigas *et al.*, 1994; Artigas *et al.*, 1996). The 5-HT_{1A} receptor has many other indicated roles, including in food intake (Gilbert *et al.*, 1988; Yamada *et al.*, 1998), memory (Edagawa *et al.*, 1998) and sexual behaviour (Maswood *et al.*, 1998).

Two groups have used the "knock out" approach in mice to examine the effects of the loss of 5-HT_{1A} receptor expression on behaviour, using models of anxiety and depression (Ramboz *et al.*, 1998; Heisler et al., 1998). Knockout animals showed no difference in the brain levels of serotonin compared with wild types and Ramboz et al. measured the amount of serotonin released following electrical stimulation in brain slices and in both wild type and knockout animals, 8-OH-DPAT was able to reduce serotonin release by 30-40%. This could suggest that either the 5-HT_{1A} receptor does not play a large part in modulating serotonin release or that its role has been taken over by another receptor subtype. In terms of behavioural differences, tests designed to measure the anxiety levels of mice showed that the homozygous mutant mice were more "anxious" than the wild type mice. Further tests used to examine the levels of depression in mice showed that the knockout mice were less "depressed" than the WT mice, supporting the idea that a lack of functional 5-HT_{1A} receptor favours a less depressed state. It is hoped that a more complete understanding of the 5-HT_{1A} receptor-G protein interaction gained from these studies may lead to advances in therapeutic treatments for a wide range of mental illness, such as depression, anxiety and eating disorders.

This work explored the interaction of the human 5-HT_{1A} G protein coupled receptor and various inhibitory G_α subunits through which the receptor has been demonstrated previously to couple to inhibition of adenylyl cyclase (Banerjee *et al.*,

1993; Fargin *et al.*, 1989; Liu and Albert, 1991; Varrault *et al.*, 1992; Kellett *et al.*, 1999). The C terminal tail of the 5-HT_{1A} receptor was fused to the N terminal tail of the G_α subunit forming a single construct (Figure 3.2). Both wild type $G_{i1\alpha}$/$G_{o1\alpha}$ and mutant (Gly^{351} and Ile^{351}) forms of these G_α subunits were utilised. These mutations have been previously characterised (Wise *et al.*, 1997; Bahia *et al.*, 1998; Jackson *et al.*, 1999) and are resistant to pertussis toxin-catalysed ADP-ribosylation, a modification which prevents the activation of these G proteins by receptors.

The isolated 5-HT_{1A} receptor and the six fusion proteins were stably expressed in HEK293 cells prior to functional analysis. Five of these cell lines were already available with only the 5-HT_{1A}/$G_{o1\alpha}$ and the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ constructs requiring stable expression. The expression levels in membranes of 48 clones of each construct were assessed by single point [^{3}H]-MPPF binding (Figures 3.3 and 3.4). MPPF is a selective 5-HT_{1A} receptor antagonist (Thielen *et al.*, 1996; Thielen and Frazer, 1995). With pseudo saturation receptor expression levels similar to the stable cell lines currently available, two clones expressing each construct were selected and complete [^{3}H]-MPPF saturation analysis carried out (Figure 3.5). These data were analysed by nonlinear regression and B_{max} calculated (Table 3.1). The two clones selected for all future signal transduction analysis were Go27 and GoI48. Table 3.3 shows the receptor expression levels of the seven stable cell lines, indicating B_{max} of cells that would be routinely pertussis toxin-treated.

A second method was used to identify positive expression of the fusion proteins. Membrane fractions of the clones selected were immunoblotted using two separate antisera. In panel A of figure 3.6, an antiserum with an epitope between amino acids 159 and 168 of $G_{i1\alpha}$ identified polypeptides of apparent Mr between 85 and 90kDa in the lanes containing membranes from cells stably expressing 5-HT_{1A} receptor fused to WT and mutant $G_{i1\alpha}$. No protein of this size was found in the lanes containing membranes of the isolated receptor or the $G_{o1\alpha}$ fusions. In panel B of figure 3.6, an antiserum with an epitope between amino acids 1-16 of $G_{o\alpha}$ identified polypeptides of apparent Mr between 85 and 90 kDa in the lanes containing membranes from the cells stably expressing fusions between the 5-HT_{1A} receptor and WT or mutant $G_{o1\alpha}$. Again, no protein of this size was found in the lanes containing membranes of the isolated receptor or the $G_{i1\alpha}$ fusion proteins.

The next step was to assess the functional activity of the 5-HT_{1A} receptor G protein fusions. Kellett *et al* (1999) previously reported that 5-HT_{1A}/$G_{i1\alpha}$ fusions produced decreases in forskolin-stimulated adenylyl cyclase activity following agonist activation. This effect was blocked by pre-treatment with pertussis toxin in cells expressing the isolated 5-HT_{1A} receptor and the WT $G_{i1\alpha}$ fusion, but no effect was observed in those expressing the Gly^{351} and Ile^{351} mutants. The result of receptor activation by 5-HT on forskolin-stimulated cAMP production was examined in intact cells expressing the 5-HT_{1A}/$G_{i1\alpha}$ and $G_{o1\alpha}$ fusions. 5-HT produced concentration dependent decreases in maximal forskolin-stimulated (50μM) adenylyl cyclase activity in cells expressing all constructs, but pre-treatment with pertussis toxin significantly inhibited this in cells containing the isolated 5-HT_{1A} receptor and both the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ receptor – G protein fusions. Pertussis toxin treatment had no effect on signal transduction in cells expressing the Gly^{351} and Ile^{351} mutants. This is an indication that the receptor-G protein fusions do not signal through the endogenous pool of inhibitory G proteins, only via the G protein fused to the receptor's C terminal tail. If endogenous G proteins were activated, a difference between vehicle and pertussis toxin pre-treated cells would be expected. The EC_{50} value (4nM) observed for inhibition of forskolin-stimulated adenylyl cyclase activation for the 5-HT_{1A} receptor was significantly different ($P < 0.05$) to those of the two WT G protein – receptor fusions ($G_{i1\alpha}$ – 48nM, $G_{o1\alpha}$ 52nM). This could indicate that the isolated receptor can activate many inhibitory G proteins and that fusion of a G protein α subunit to the C terminal tail of the 5-HT_{1A} receptor prevents its coupling to the endogenous pool of inhibitory G proteins.

For the 5-HT_{1A} receptor and both the 5-HT_{1A}/$G_{i1\alpha}$ and the 5-HT_{1A}/$G_{o1\alpha}$ fusion proteins, pertussis toxin treatment of the cells results in abolition of agonist mediated inhibition of cAMP production (Figure 3.7). Examining the EC_{50} values of 5-HT for the $G_{i1\alpha}$ fusion proteins in greater detail (Table 3.3), the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion has the greatest potency to inhibit cAMP production (1.5 +/- 0.1nM non, and 1.9 +/- 0.4nM pertussis toxin-treated respectively). These values are significantly lower than the EC_{50} values for the Gly^{351} $G_{i1\alpha}$ mutant ($P < 0.05$). The trend among the $G_{i1\alpha}$-receptor fusion proteins following 5-HT stimulation is for the Gly^{351} mutant to have the weakest potency followed by the WT Cys^{351}, and the most potent being the Ile^{351} mutant. This result is supported by the findings of Bahia *et al.* (1998) where Cys^{351}

in the $G_{i1\alpha}$ protein was mutated to the 19 other naturally occurring amino acids. They found that the EC_{50} in agonist induced GTPγS binding, following stimulation of the α_{2A}-adrenoceptor, to the Cys^{351} mutants correlated strongly (r = 0.92) with the partition coefficient between octanol/water (log P(octanol/water)) following the elimination of methionine and proline. The reason for methionine being an outlier was suggested that it might be present as methionine sulfoxide which would severely affect its hydrophobicity, and proline may affect the conformation of the C terminal tail of the G protein. In short, they concluded that the more hydrophobic the amino acid at position^{351} in the α subunit of inhibitory G proteins, the stronger the receptor-G protein interaction.

This trend is not as apparent in intact cell measures of regulation of adenylyl cyclase activity by the 5-HT_{1A} receptor-$G_{o1\alpha}$ proteins (Table 3.4). Although the Ile^{351} mutant had the lowest EC_{50} value, the WT $G_{o1\alpha}$ fusion had the highest. No firm conclusions can be drawn from these data as statistical analysis failed to show that any of the EC_{50} values were significantly different from one another.

Comparison of the $G_{i1\alpha}$ fusion protein results to those for the $G_{o1\alpha}$ fusions showed an obvious trend towards the fusion proteins between the 5-HT_{1A} receptor and the WT $G_{i1\alpha}$ protein and its mutants having a greater ability to inhibit forskolin stimulated adenylyl cyclase activity. The EC_{50} for inhibition of cAMP production by the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein of 2nM was significantly less than that of the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion ($P < 0.05$). This finding is supported by work carried out in a variety of expression systems. Addition of purified G protein subunits to Sf9 cell membranes expressing 5-HT_{1A} receptors demonstrated that the ability of G_{α} subunits to shift receptors into a high affinity state for agonists in the presence of either brain, or retinal β_1/γ was greatest with α_{i1} followed by α_{i2}, α_{i3} and finally α_o (Clawges *et al.*, 1997). 5-HT_{1A} receptors expressed in *E.Coli* showed a rank order of affinity for reconstituted purified mammalian G protein α subunits of $G_{i3\alpha} > G_{i2\alpha} > G_{i1\alpha} >> G_{o\alpha} >> G_{s\alpha}$ (Bertin *et al.*, 1992). Finally, agonist promoted physical coupling of the 5-HT_{1A} receptor to G proteins was demonstrated in HeLa and CHO cells using high affinity binding and co-immunoprecipitation assays with an apparent rank order of $G_{i3\alpha} > G_{i2\alpha} > G_{i1\alpha} \cong G_{o1\alpha} > G_{z\alpha} >> G_{s\alpha}$ (Garnovskaya *et al.*, 1997; Raymond *et al.*, 1993).

These results do not indicate if the inhibition of cAMP production is via the G_α or $G_{\beta\gamma}$ subunits as pertussis toxin prevents agonist mediated receptor activation of the G protein. One approach to investigate the mediator of the effects of the 5-HT_{1A} receptor on adenylyl cyclase activity was to co-express to the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ receptor and transducin protein α subunit in HEK293 cells (Figure 3.10). As this work is in transiently transfected cells where only a fraction of the cells express the constructs, it was not possible to use forskolin to stimulate adenylyl cyclase activity, so the G_s linked β_2 receptor is co-transfected into the cells and the β_2 agonist, isoprenaline used to stimulate activity. In theory, the over-expression of transducin protein α subunit could restrict activation of second messenger systems by $G_{\beta\gamma}$ subunits by binding to the $\beta\gamma$ subunits once they are released from the 5-HT_{1A} receptor-fused G protein heterotrimer following 5-HT activation of the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein.

No significant difference was found between the EC_{50} values for inhibition of adenylyl cyclase activity in cells either transfected with or not transfected with transducin α (3.9nM and 4.3nM respectively), which may indicate that agonist activation of the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein results in inhibition of adenylyl cyclase activity via the G_α proteins. However, a western blot examination of the cells transfected with transducin α was not performed to confirm overexpression of the transducin α protein.

Another noticeable characteristic of the whole cell cyclase inhibition curves for the 5-HT_{1A} receptor $G_{i1\alpha}$ protein fusions is that the Hill slope gets steeper from the Gly^{351} through the Cys^{351} to the Ile^{351}. Statistical analysis of slope values for the pertussis toxin treated Gly^{351} (-1.075+/-0.13) and Ile^{351} (-1.791+/-0.01) mutants showed them to be significantly different ($P < 0.05$ (0.0055)). This may be related to the hydrophobicity of the amino acid at position^{351} discussed above and a reflection of the degree of intrinsic constitutive activity imparted on the receptor following G protein fusion. Indications of intrinsic constitutive activity in 5-HT_{1A} receptor-mutant G protein fusions were suggested by Kellett *et al.* (1999). Spiperone, which acts as an inverse agonist at the 5-HT_{1A} receptor (Barr and Manning 1997; Newman-Tancredi *et al.*, 1997a,b), had no effect on GTPase activity of the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ mutant, but functioned effectively in membranes expressing the Ile^{351} mutant. Prior treatment with pertussis toxin had no effect on these data, demonstrating that the

inverse agonist effect could only be through the mutant $G_{i1\alpha}$ protein fused to the 5-HT_{1A} receptor and not the endogenous pool of G proteins. This confirms that the constitutive activity was a result of interaction between the 5-HT_{1A} receptor and the fused mutant G protein.

The final point of discussion raised by the results was the presence of a 5-HT induced stimulation of cAMP production at concentrations greater than 1μM. This effect was most obvious in the pertussis toxin-pretreated data from the 5-HT_{1A} stable cell line (Figure 3.7) and from both pertussis toxin-pretreated and untreated data for the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ (Figure 3.8) stable cell line. Malmberg and Strange (2000), who observed a similar effect when measuring adenylyl cyclase activity in WT and mutant 5-HT_{1A} receptors, attributed this to coupling of the 5-HT_{1A} receptor to G_s. As they only saw the effect with 5-HT and not with the 5-HT_{1A} selective agonist 8-OH-DPAT and partial 5-HT_{1A} agonist buspirone, it seems more likely that it could be due to low level expression of a G_s-linked 5-HT receptor of a different subtype. This is supported by the results shown in figures 3.9 and 3.10. A whole cell adenylyl cyclase assay was carried out on non transfected HEK293 cells. In figure 3.9 where adenylyl cyclase activity was not stimulated with forskolin, concentrations of 5-HT greater than 1μM produced elevations in cAMP production approximately three fold over basal. Following forskolin stimulation this effect was still recorded. At 50μM forskolin, 5-HT still more than doubles this effect (Figure 3.10). Forskolin is regarded as a non-specific stimulator of all forms of adenylyl cyclase. For 5-HT may be producing stimulation above these effects through two possible mechanisms. It may be that forskolin is only a partial activator of some or all forms of adenylyl cyclase and that 5-HT is activating a receptor that produced complete activation. This hypothesis could be investigated by checking if a $G_{s\alpha}$ linked receptor such as the β_2-adrenoceptor could stimulate adenylyl cyclase activity above maximal forskolin activation. A second possibility is that 5-HT is activating a second messenger system that results in modification of adenylyl cyclase leading to a more active form of the enzyme. This could possibly be following PKC/PKA mediated phosphorylation of adenylyl cyclase, a mechanism that could be investigated using PKC/PKA inhibitors such as H-7 or H-9.

Taken together with the lack of stimulation of adenylyl cyclase by 8-OH-DPAT, the data presented suggest that this effect is not mediated through the 5-HT_{1A}

receptor. The other possible candidates for the role that have previously been shown to be G_s linked, are 5-HT_4 (Gerald *et al.*, 1995; Claeysen *et al.*, 1996; Van den Wyngaert *et al.*, 1997), 5-HT_{5A}, 5-HT_6 (Monsma *et al.*, 1993; Ruat *et al.*, 1993a,b; Kohen *et al.*, 1996; Boess *et al.*, 1997; Schoeffter and Waeber, 1994; Sebben *et al.*, 1994; Unsworth and Molinoff, 1993; Conner and Mansour, 1990) and 5-HT_7 (Bard *et al.*, 1993; Lovenberg *et al.*, 1993a,b; Plassat *et al.*, 1993; Ruat *et al.*, 1993a,b; *Shen et al.*, 1993; Tsou *et al.*, 1994; Hirst *et al.*, 1997; Heidmann *et al.*, 1997, 1998; Stam *et al.*, 1997). As 8-OH-DPAT is a weak agonist at the 5-HT_7 receptor (Wood *et al.*, 2000) and does not produce any cyclase stimulation in HEK293 cells (Malmberg and Strange, 2000), it can be excluded. Another candidate, the 5-HT_{5A} receptor, has been show to couple to pertussis toxin sensitive G proteins, i.e. $G_{i/o}$, and to inhibit adenylyl cyclase activity in HEK293 cells (Francken *et al.*, 1998). Thus the most likely candidate is either 5-HT_4 or 5-HT_6 at low levels, or possibly another endogenous receptor that has low affinity for 5-HT for example the β_2-adrenergic receptor that is endogenously expressed in HEK293 cells. It is possible to uncouple signalling through G_s proteins by pre-treatment with cholera toxin. This would not be appropriate in this assay, as cholera toxin restricts the G_s protein to the GTP bound form, resulting in constant stimulation of second messenger systems.

Following the results of the whole cell adenylyl cyclase assays, it was decided that the affinity of the 5-HT_{1A} receptor - mutant Gly^{351} and Ile^{351} G protein fusions should be investigated further using a variety of radioligand binding assays.

Chapter 4

Radioligand Binding Assays

4.1 Introduction

G protein - coupled receptors exist in different states that have a varied degree of affinity for agonists, but similar high affinity for antagonists. Existence of the receptor in the G protein coupled form imparts a high degree of affinity for agonists, but dissociation of the G protein results in free receptor with a low degree of affinity which can often no longer be labelled by agonists (Emerit *et al.*, 1990). The binding of antagonists is independent of the G protein - coupled state of the receptor, as they bind either form with equivalent affinity (Kobilka, 1992). The difference in affinity for a compound for these affinity states can give an indication of a receptor's intrinsic activity (Birdsall and Lazareno, 1997). Given the indications that some of the 5-HT_{1A} receptor G protein constructs introduced in chapter three may have constitutive activity and altered receptor – G protein affinities, it was decided to investigate this further using a radioligand binding approach.

GPCR signalling is initiated by the formation of a ternary complex between a ligand, receptor and a G protein (Hepler and Gilman, 1992; Neubig *et al.*, 1988). Once a receptor binds an agonist, it shifts into an activated state and associates with its cognate G protein. In this activated complex, the agonist is bound with high affinity. Binding of an activated receptor to a G protein causes a conformational change in the G_α subunit, reducing the affinity of the G protein for GDP, resulting in the release of GDP and the binding of GTP (Brandt and Ross, 1986). Conversely, an excess of GDP lowers the affinity of the G protein for the receptor (Hepler and Gilman, 1992). In a radioligand binding assay, increasing concentrations of GDP result in the dissociation of receptor and G protein, indicated by a decrease in specific binding of the agonist to the receptor (Waldhoer *et al.*, 1999). Waldhoer *et al* (1999) showed a decrease in high affinity agonist binding to adenosine A_1 receptor G_α subunit fusions. Unlike previous studies using α_{2A}-adrenoreceptor – G protein fusions where mutation of Cys^{351} in the α subunit of the G protein resulted in decreased affinity of the G protein for the receptor with Gly^{351} and increased affinity with Ile^{351} (Bahia et al., 1998), Waldhoer *et al* (1999) found that both Gly and Ile substitutions at Cys^{351} decreased the affinity of the G protein for the receptor.

In order to investigate any changes in affinity of mutated G_i and G_o α subunits for the 5-HT_{1A} receptor, two main approaches were used. Firstly, the affinity of the agonist [^{3}H]-8-OH-DPAT for the 5-HT_{1A} receptor and the $G_{i1\alpha}$ and

$G_{o1\alpha}$ fusion proteins was assessed using GDP prevention bindings. Secondly, the ability of suramin to inhibit the binding of [^{3}H]-8-OH-DPAT was examined. Suramin binds directly to G protein α subunits and can be used to estimate the affinity of G protein for receptor by competing with the activated receptor for G protein binding (Waldhoer *et al.*, 1998; Freissmuth *et al.*, 1996).

4.2 Results

The affinity of the 5-HT_{1A} selective agonist [^{3}H]-8-OH-DPAT for the 5-HT_{1A} receptor and 5-HT_{1A} receptor – G protein fusions was examined in GDP prevention binding assays. Increasing concentrations of GDP from 10nM to 1mM displaced the binding of [^{3}H]-8-OH-DPAT in a concentration dependent manner (Figures 4.1 – 4.3). Pertussis toxin treatment of cells prior to membrane production and assaying for binding abolished any high affinity binding of the agonist radioligand to the 5-HT_{1A} receptor or 5-HT_{1A} receptor fused to the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ G proteins (Figure 4.1). The EC_{50} for GDP prevention of binding to membranes expressing the 5-HT_{1A} receptor was 13.7 +/- 2.4μM (Table 4.1) and the values for membranes expressing the 5-HT_{1A}/$G_{i1\alpha}$ and 5-HT_{1A}/$G_{o1\alpha}$ fusion proteins were 2.2 +/- 0.5 and 5.9 +/- 3.8μM respectively (Tables 4.1 and 4.2). The EC_{50} for the 5-HT_{1A} receptor was not significantly different to that of the 5-HT_{1A}/$G_{o1\alpha}$ fusion, but was significantly different to the 5-HT_{1A}/$G_{i1\alpha}$ fusion ($P < 0.05$). The lower EC_{50} values for the 5-HT_{1A}/$G_{i1\alpha}$ and 5-HT_{1A}/$G_{o1\alpha}$ fusion proteins may reflect the lack of coupling to the endogenous G protein pool, restricting coupling to its fused G protein. The higher EC_{50} value of the 5-HT_{1A} receptor in this assay may reflect the effects of a greater number of instances of coupling of endogenous G proteins to the receptor, putting it in the high affinity binding state for a greater period of time.

GDP prevented specific binding of [^{3}H]-8-OH-DPAT to membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}$G/I fusions in a concentration dependent manner (Figure 4.2). Pertussis toxin treatment of the cells prior to harvesting and membrane production abolished any potential binding of the fusions with the endogenous pool of G_i proteins as ADP-ribosylation of Cys^{351} prevents receptor coupling. This pre-treatment did not have any effect on the EC_{50} values of GDP prevention for either construct ($P > 0.05$) (Table 4.1). A lack of coupling of the pertussis toxin-resistant receptor - G protein fusions to endogenous G proteins was also indicated in the adenylyl cyclase results in Chapter 3. The EC_{50} values of either the non - pertussis toxin treated 5-HT_{1A}/$G_{i1\alpha}C^{351}$G and 5-HT_{1A}/$G_{i1\alpha}C^{351}$I fusions (0.30 +/- 0.05 and 10.0 +/- 3.1μM respectively), or the pertussis toxin pretreated fusions (0.10 +/- 0.05 and 6.9 +/- 2.9μM respectively) were not statistically different from each other ($p = 0.0593$) (Table 4.1). A rank order of EC_{50} values for the constructs of the Ile^{351} (approx. 7-10μM) construct, followed by the WT Cys^{351} $G_{i1\alpha}$ fusion (2.2μM) and

lastly by the Gly^{351} mutant (approx. 0.15-0.25μM), indicates that the receptor has highest affinity for the G protein with Ile at position^{351} followed by WT Cys and has poorest affinity for the G protein with Gly at position^{351}.

GDP prevented [^{3}H]-8-OH-DPAT binding to the 5-HT_{1A}/$G_{o1\alpha}C^{351}$G/I fusions in a concentration-dependent manner and, as with the $G_{i1\alpha}$ mutants, pertussis toxin pre-treatment did not have any effect on agonist binding (Figure 4.3). Neither the EC_{50} values for the non pertussis toxin or the pertussis toxin pre-treated Ile^{351} mutant (3.0 +/- 0.6 and 2.6 +/- 0.6μM respectively), nor the non pertussis toxin or pertussis toxin-pretreated Gly^{351} mutant (0.10 +/- 0.04 and 0.10 +/- 0.04μM respectively), were statistically different from the EC_{50} value for the WT Cys^{351} 5-HT_{1A} receptor-G protein fusion (5.9 +/- 3.8μM) due in part to the large error of the latter EC_{50} (Table 4.2). The EC_{50} values for the non-pertussis toxin and pertussis toxin-pretreated 5-HT_{1A}/$G_{i1\alpha}C^{351}$G/I mutants were statistically different from one another ($P < 0.05$). As with the $G_{i1\alpha}$ fusion proteins this could indicate that the G protein with Ile^{351} has a higher affinity for the 5-HT_{1A} receptor, with the Cys^{351} having identical affinity, but the Gly^{351} mutant having a lower affinity for the 5-HT_{1A} receptor.

Comparison of EC_{50} values of GDP prevention of [^{3}H]-8-OH-DPAT binding of the pertussis toxin-treated 5-HT_{1A}/$G_{i1\alpha}C^{351}$G to the 5-HT_{1A}/$G_{o1\alpha}C^{351}$G mutants did not show a statistically significant difference ($P > 0.05$). Neither were the EC_{50} values of the 5-HT_{1A}/$G_{i1\alpha}C^{351}$I or the 5-HT_{1A}/$G_{o1\alpha}C^{351}$I mutants significantly different ($P > 0.05$). However, the trend in higher EC_{50} values for GDP displacement of agonist binding for the $G_{i1\alpha}$ fusions compared to their $G_{o1\alpha}$ counterparts, could indicate that $G_{i1\alpha}$ had higher affinity for the 5-HT_{1A} receptor than $G_{o1\alpha}$.

The effect of increasing concentrations of GDP on the binding of the 5-HT_{1A} selective antagonist [^{3}H]-MPPF was investigated using the same binding protocol. Antagonists do not differentiate between the high and low affinity states of receptor binding, but bind to both with equal affinity, so it is unsurprising that neither prior treatment with pertussis toxin, nor increasing concentrations of GDP, had any effect on the binding of this radioligand (Figures 4.4, and 4.5). In figure 4.4, the binding of [^{3}H]-MPPF to the 5-HT_{1A}/$G_{i1\alpha}$ fusion was unaffected by either increasing concentrations of GDP or prior pertussis toxin treatment of the cells which ADP ribosylated the entire pool of endogenous G_i plus the $G_{i1\alpha}$ protein fused to the 5-

HT_{1A} receptor. Again neither GDP nor pertussis toxin pretreatment had any effect of the binding of [^{3}H]-MPPF to the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion (Figure 4.5).

The affinity of the 5-HT_{1A} receptor for the selection of G proteins was further investigated using the compound suramin. Suramin inhibits the formation of the ternary ligand-receptor-G protein complex by competing directly with the receptor for G protein binding (Huang *et al.*, 1990; Hohenegger *et al.*, 1998; Beindl *et al.*, 1996). Its use can give an indication of a G protein's affinity for an agonist-bound receptor (Waldhoer *et al.*, 1999).

In figure 4.6, the effects of increasing concentrations of suramin on the binding of the agonist [^{3}H]-8-OH-DPAT to the 5-HT_{1A} receptor and the 5-HT_{1A} receptor-$G_{i1\alpha}$ and $G_{o1\alpha}$ protein constructs was investigated following preparation of membranes from cells stably expressing the constructs. As in the GDP prevention bindings, pre-treatment of the cells prior to harvesting with pertussis toxin uncouples the receptor from the fused and endogenous pool of G proteins (Figure 4.6), preventing binding of the radioligand agonist except with low affinity. Suramin produced a concentration dependent decrease in binding of [^{3}H]-8-OH-DPAT to the 5-HT_{1A} receptor and its WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusions presumably by uncoupling the receptor-G protein interaction. Statistically there was not a significant difference between the EC_{50} values for the proteins, with an EC_{50} for the 5-HT_{1A} receptor of 1.8 +/- 0.8μM and of 1.7 +/- 0.5 and 1.3 +/- 0.6μM for the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins respectively (Tables 4.3 and 4.4).

As in the GDP prevention binding assays, pertussis toxin treatment of cells prior to harvesting and membrane production had no significant effect on the binding of [^{3}H]-8-OH-DPAT to membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}$G/I fusions (Figure 4.7). Suramin inhibited binding of the agonist to the receptors in a concentration dependent manner, with EC_{50} values for the non- and pertussis toxin pre-treated Gly^{351} mutant of 1.2 +/- 0.2 and 0.8 +/- 0.1μM respectively, and 4.8 +/- 0.8 and 5.8 +/- 0.4μM for the Ile^{351} mutant, non- and pertussis toxin-treated respectively (Table 4.3). The EC_{50} values for the Gly^{351} mutant +/- pertussis toxin were not significantly different from each other ($P > 0.05$) and neither were those of the Ile^{351} mutant +/- pertussis toxin ($P > 0.05$). However, the EC_{50} values of either the non pertussis toxin treated Gly^{351} and Ile^{351} mutants, or the pertussis toxin treated Gly^{351} and Ile^{351} mutants, were significantly different from each another ($P < 0.05$)(P

= 0.0118 and 0.0002 respectively). As in previous assays the results indicate a rank order of affinity of suramin to prevent G protein binding of agonist bound receptor, with suramin having the highest affinity with Ile^{351} (5-6μM approx.) mutants, followed by the WT Cys^{351} (1.7μM approx.), and lastly by Gly^{351} (0.8-1.2μM approx.). Therefore, the greater the concentration of suramin required to produce a 50% reduction in agonist binding, the greater the affinity the G protein has for the agonist-bound receptor.

Similar results were measured in the suramin prevention bindings of the membranes expressing the 5-HT_{1A}/$G_{o1\alpha}C^{351}$G/I fusion proteins. Again treatment of the cells with pertussis toxin prior to harvesting and membrane production did not have a significant effect on the binding of [^{3}H]-agonist to the receptor-G protein constructs ($P > 0.05$) (Figure 4.8). Suramin produced a concentration dependent decrease in agonist binding, reaching a maximal effect in the 5-HT_{1A}/$G_{o1\alpha}C^{351}$G fusion at approximately 10μM, but not having reached a maximal effect on the 5-HT_{1A}/$G_{o1\alpha}C^{351}$I fusion even at 100μM. The EC_{50} values for the non-, and pertussis toxin-treated membranes were not significantly different for the Gly^{351} or the Ile^{351} mutants. On comparison of the EC_{50} values for the different mutants to each other, the non-pertussis toxin treated values were significantly different from one another ($P = 0.0142$), as were the pertussis toxin-treated values ($P = 0.0003$) (Table 4.4). Again this would indicate that in the suramin prevention binding assay, the Ile^{351} G protein had a higher affinity for the agonist bound 5-HT_{1A} receptor than the Gly and Cys^{351} G proteins, that appeared to have equal affinity for the receptor.

As a control, the effect of increasing concentrations of suramin on the binding of the 5-HT_{1A} selective antagonist [^{3}H]-MPPF was investigated. Given that antagonists do not distinguish between the high and low affinity binding conformations of a GPCR, binding to both equally, suramin had no effect on the binding of [^{3}H]-MPPF to the isolated receptor (Figure 4.9) or 5-HT_{1A} receptor-G protein fusions (Figure 4.10). This demonstrates that suramin is not competing directly with [^{3}H]-MPPF or [^{3}H]-8-OH-DPAT at the ligand binding site.

In order to examine the proportion of a receptor that is present at rest in the high affinity (G protein coupled)(%R_H) and the low affinity (G protein uncoupled)(%R_L) states, agonist displacement of [^{3}H]-antagonist binding was examined for the 5-HT_{1A}/$G_{i1\alpha}C^{351}$G/I fusions (Figures 4.11 and 4.12). Biphasic

competition curves were generated with the first EC_{50} corresponding to the high affinity site for the agonist and the second EC_{50} corresponding to the low affinity site for the agonist. The antagonist [^{3}H]-MPPF bound to these sites with equal high affinity, and using increasing concentrations of 8-OH-DPAT, the antagonist was displaced firstly from the high affinity agonist binding site and secondly from the low affinity site. Comparison of the results from this binding allows the calculation of the proportion of the receptor present in the high and low affinity states (Table 4.5). The important data here are the pertussis toxin treated data as this allows us to look only at the influence of the G protein fused to the 5-HT_{1A} receptor. Although there is not statistical difference in the EC_{50} values for the Gly^{351} and Ile^{351} pertussis toxin treated samples, there is a significant difference between the fractions of the constructs in the high affinity state ($P < 0.05$ (0.0039)). There were over twice as many Ile^{351} receptor-G protein fusion proteins in the high affinity state ($\%R_{II}$) as there are Gly^{351} fusion proteins in the high affinity state.

As a control, binding of the 5-HT_{1A} selective agonist [^{3}H]-8-OH-DPAT to non-transfected HEK293 membranes was examined (Figure 4.13). Over a period of sixty minutes, no specific binding of the radioligand could be detected.

Figure 4.1 **Inhibition of agonist radioligand binding to membranes prepared from HEK293 cells stably expressing the 5-HT_{1A} receptor and the 5-HT_{1A} receptor-$G_{i1\alpha}$ and $G_{o1\alpha}$ protein fusions by GDP**

Percentage of specific [^{3}H]-8-OH-DPAT binding in the absence (100%) or presence of increasing concentrations of GDP in HEK293 cells stably expressing the 5-HT_{1A} receptor and the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ protein fusions. Pertussis toxin-treatment and increasing concentrations of GDP result in reduced binding of the agonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

- 5-HT_{1A}
- 5-HT_{1A} ptox
- 5-$HT_{1A}/G_{i1\alpha}$
- 5-$HT_{1A}/G_{i1\alpha}$ ptox
- 5-$HT_{1A}/G_{o1\alpha}$
- 5-$HT_{1A}/G_{o1\alpha}$ ptox

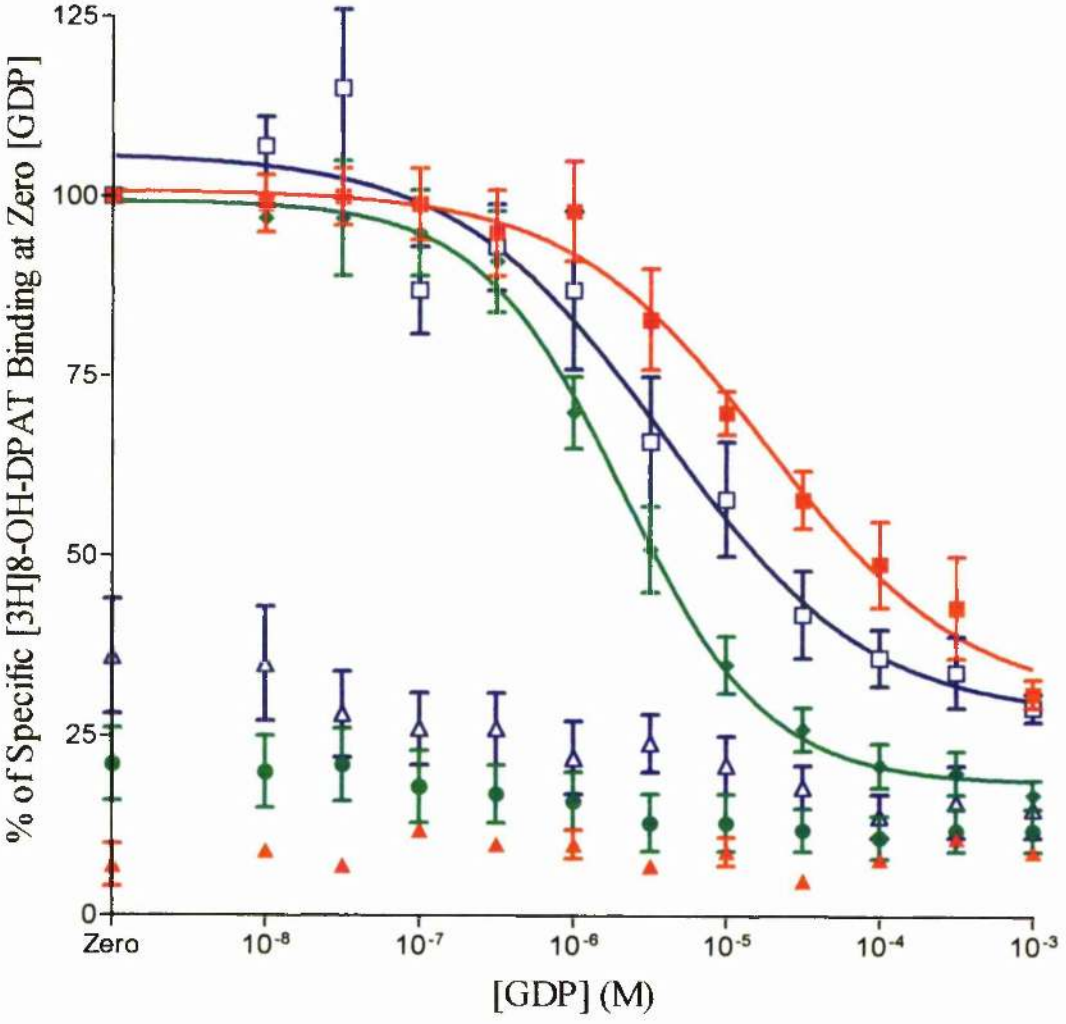

% of Specific [3H]8-OH-DPAT Binding at Zero [GDP]
125
100
75
50
25
0
Zero
10-8
10-7
10-6
10-5
10-4
10-3
[GDP] (M)

Figure 4.2 **Inhibition of agonist radioligand binding by GDP, to membranes prepared from HEK293 cells stably expressing the pertussis toxin resistant 5-$HT_{1A}/G_{i1\alpha}C^{351}G/I$ fusion proteins**

Percentage of specific [^{3}H]-8-OH-DPAT binding in the absence (100%) or presence of increasing concentrations of GDP in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{i1\alpha}$ protein fusions with Gly^{351} and Ile^{351} mutations. Increasing concentrations of GDP result in reduced binding of the agonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.


5-$HT_{1A}/G_{i1\alpha}C^{351}G$


5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox


5-$HT_{1A}/G_{i1\alpha}C^{351}I$


5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox

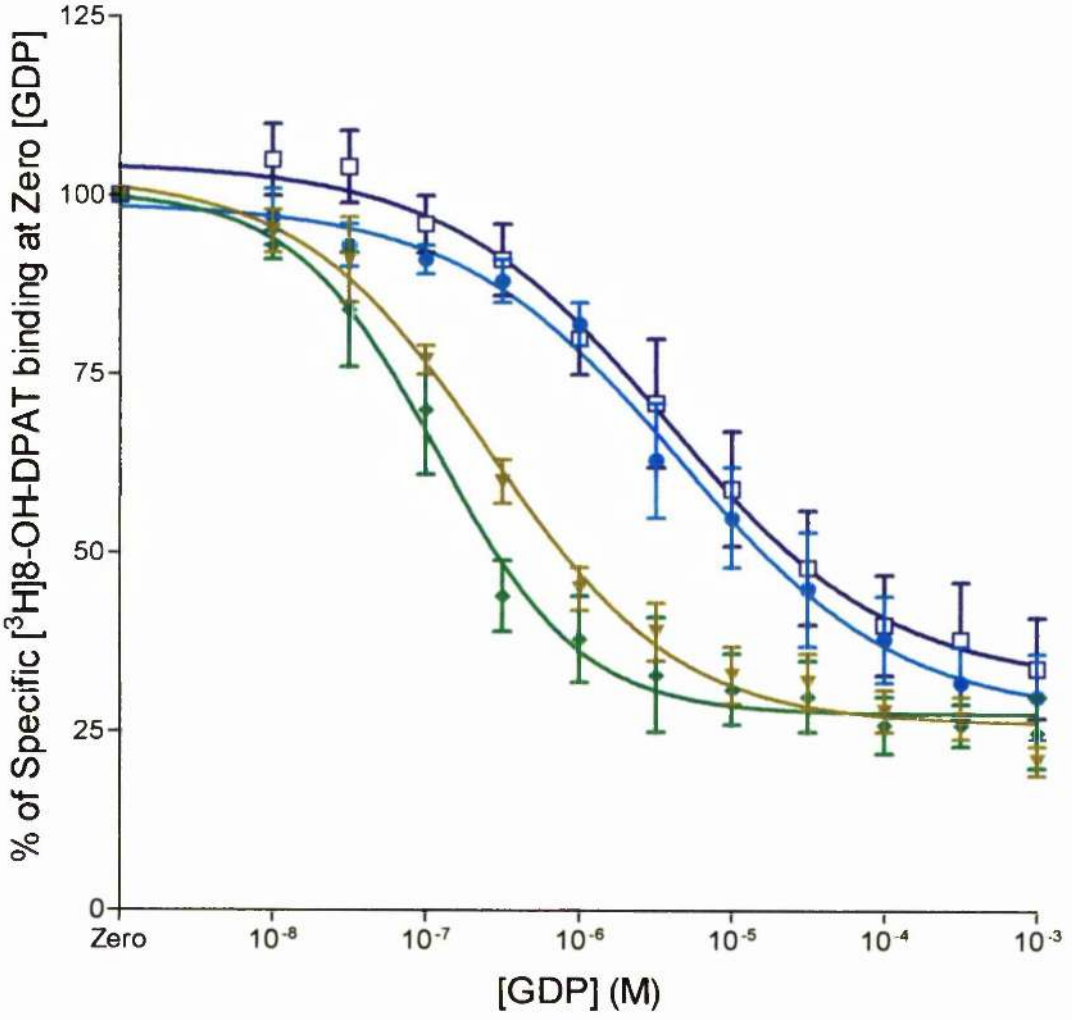

% of Specific [3H]8-OH-DPAT binding at Zero [GDP]
125
100
75
50
25
0
Zero
10-8
10-7
10-6
10-5
10-4
10-3
[GDP] (M)

Table 4.1 **Mean EC_{50} and Hill slope values for GDP prevention of [^{3}H]-8-OH-DPAT binding to membranes prepared from HEK293 cells stably expressing the 5-HT_{1A} receptor and its $G_{i1\alpha}$ fusion proteins**

Table showing the mean +/- SEM EC_{50} and Hill slope values for GDP prevention of specific [^{3}H]-8-OH-DPAT binding to the 5-HT_{1A} receptor, its WT $G_{i1\alpha}$ fusion protein and the pertussis toxin resistant $G_{i1\alpha}C^{351}G/I$ fusion proteins. Values are means of results from experiments repeated a minimum of three times.

Construct Expressed	EC_{50} (μM) Mean +/-SEM	Hillslope Mean +/- SEM	Number of Replicates
5-HT_{1A}	13.7 +/- 2.4	-0.6 +/- 0.1	4
5-$HT_{1A}/G_{i1\alpha}$	2.2 +/- 0.5	-0.9 +/- 0.1	4
5-$HT_{1A}/G_{i1\alpha}C^{351}G$	0.30 +/- 0.05	-0.7 +/- 0.1	3
5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox	0.10 +/- 0.05	-1.0 +/- 0.2	3
5-$HT_{1A}/G_{i1\alpha}C^{351}I$	10.0 +/- 3.1	-0.8 +/- 0.2	5
5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox	6.9 +/- 2.9	-0.6 +/- 0.1	5

Figure 4.3 **Inhibition of agonist radioligand binding by GDP, to membranes prepared from HEK293 cells stably expressing the pertussis toxin resistant 5-$HT_{1A}/G_{o1\alpha}C^{351}G/I$ fusion proteins**

Percentage of specific [^{3}H]-8-OH-DPAT binding in the absence (100%) or presence of increasing concentrations of GDP in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{o1\alpha}$ protein fusions with Gly^{351} and Ile^{351} mutations. Increasing concentrations of GDP result in reduced binding of the agonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times..


5-$HT_{1A}/G_{o1\alpha}C^{351}G$


5-$HT_{1A}/G_{o1\alpha}C^{351}G$ ptox


5-$HT_{1A}/G_{o1\alpha}C^{351}I$


5-$HT_{1A}/G_{o1\alpha}C^{351}I$ ptox

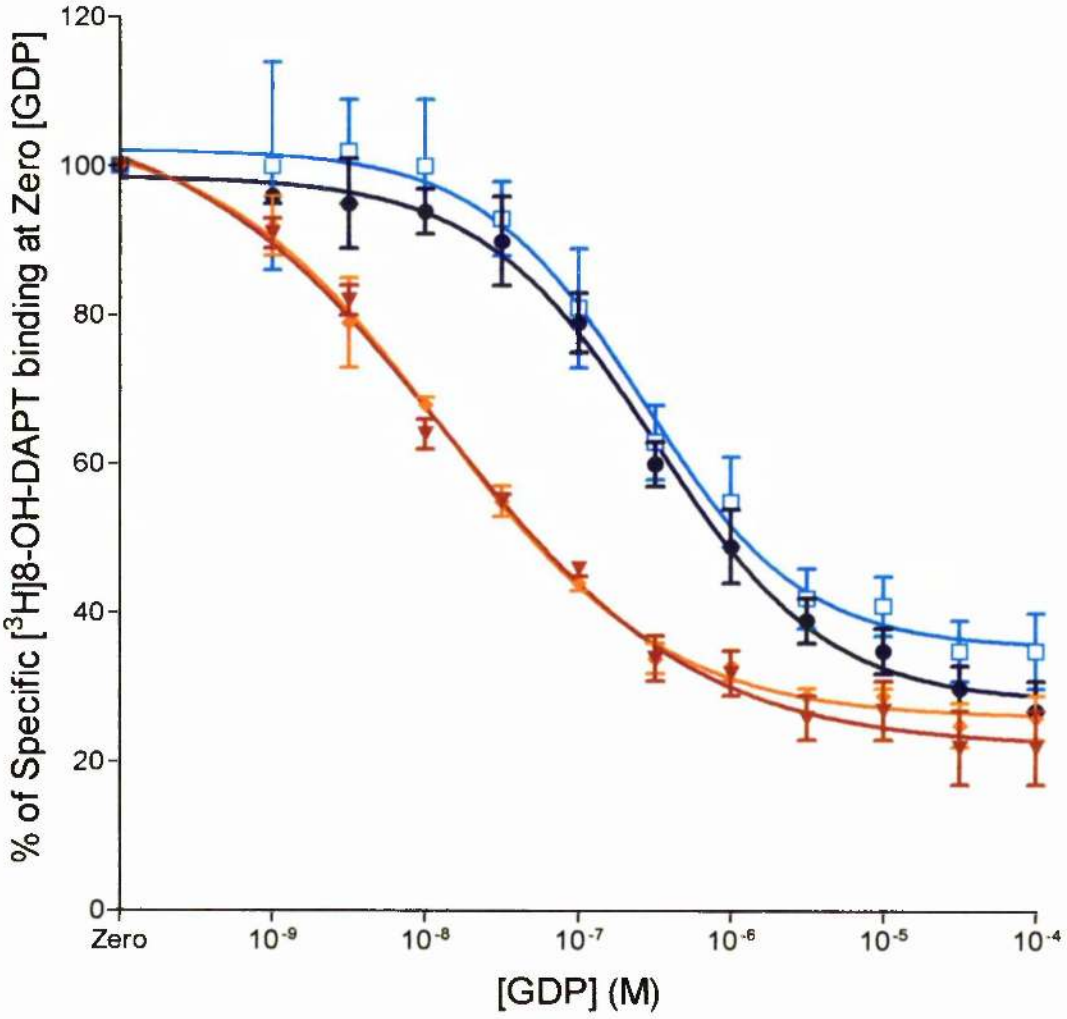

% of Specific [3H]8-OH-DAPT binding at Zero [GDP]
120
100
80
60
40
20
0
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[GDP] (M)

Table 4.2 **Mean EC_{50} and Hill slope values for GDP prevention of [^{3}H]-8-OH-DPAT binding to membranes prepared from HEK293 cells stably expressing the 5-HT_{1A} receptor and its $G_{o1\alpha}$ fusions proteins**

Table showing the mean +/- S.E.M. EC_{50} and Hill slope values for GDP prevention of specific [^{3}H]-8-OH-DPAT to the 5-HT_{1A} receptor, its WT $G_{o1\alpha}$ fusion protein and the pertussis toxin-resistant $G_{o1\alpha}C^{351}G/I$ fusion proteins. Values are means of results from experiments repeated a minimum of three times.

Construct Expressed	EC_{50} (μM) Mean +/-SEM	Hillslope Mean +/- SEM	Number of Replicates
5-HT_{1A}	13.7 +/- 2.4	-0.6 +/- 0.1	4
5-$HT_{1A}/G_{o1\alpha}$	5.9 +/- 3.8	-0.5 +/- 0.2	4
5-$HT_{1A}/G_{o1\alpha}C^{351}G$	0.10 +/- 0.04	-0.6 +/- 0.1	3
5-$HT_{1A}/G_{o1\alpha}C^{351}G$ ptox	0.10 +/- 0.04	-0.6 +/- 0.1	3
5-$HT_{1A}/G_{o1\alpha}C^{351}I$	3.0 +/- 0.6	-0.8 +/- 0.1	3
5-$HT_{1A}/G_{o1\alpha}C^{351}I$ ptox	2.6 +/- 0.6	-0.8 +/- 0.1	3

Figure 4.4 **Lack of inhibition of antagonist radioligand binding by GDP to membranes prepared from HEK293 cells stably expressing the pertussis toxin sensitive 5-$HT_{1A}/G_{i1\alpha}$ fusion protein**

Percentage of specific [^{3}H]-MPPF binding in the absence (100%) or presence of increasing concentrations of GDP in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{i1\alpha}$ protein fusion. Neither pertussis toxin-treatment, nor increasing concentrations of GDP affect binding of the antagonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

5-$HT_{1A}/G_{i1\alpha}$

5-$HT_{1A}/G_{i1\alpha}$ ptox

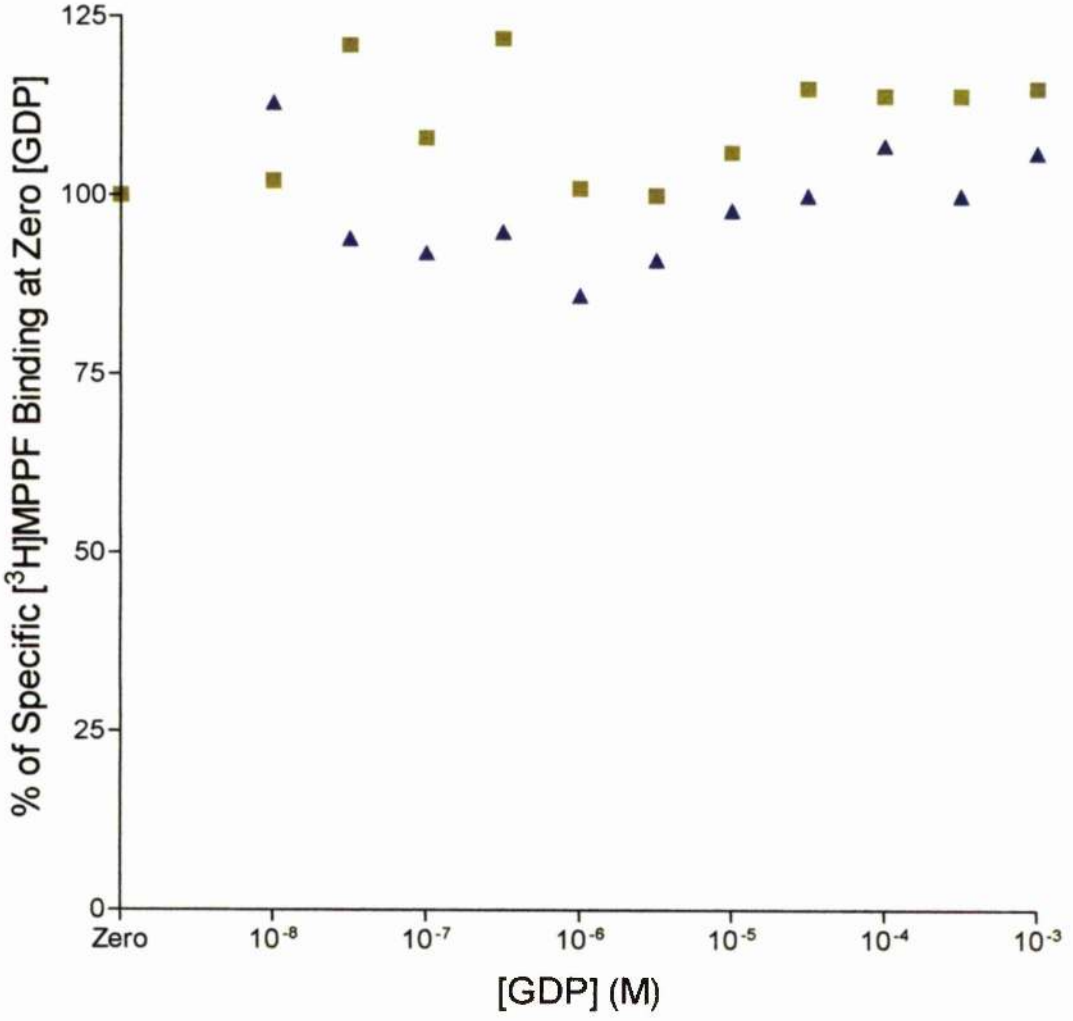

% of Specific [3H]MPPF Binding at Zero [GDP]
125
100
75
50
25
0
Zero
10-8
10-7
10-6
10-5
10-4
10-3
[GDP] (M)

Figure 4.5 **Lack of inhibition of antagonist radioligand binding by GDP to membranes prepared from HEK293 cells stably expressing the pertussis toxin-resistant 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion protein**

Percentage of specific [^{3}H]-MPPF binding in the absence (100%) or presence of increasing concentrations of GDP in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{o1\alpha}$ protein fusion with the Ile^{351} mutation. Neither pertussis toxin-treatment, nor increasing concentrations of GDP alter binding of the antagonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

5-HT_{1A}/$G_{i1\alpha}C^{351}I$

5-HT_{1A}/$G_{i1\alpha}C^{351}I$ ptox

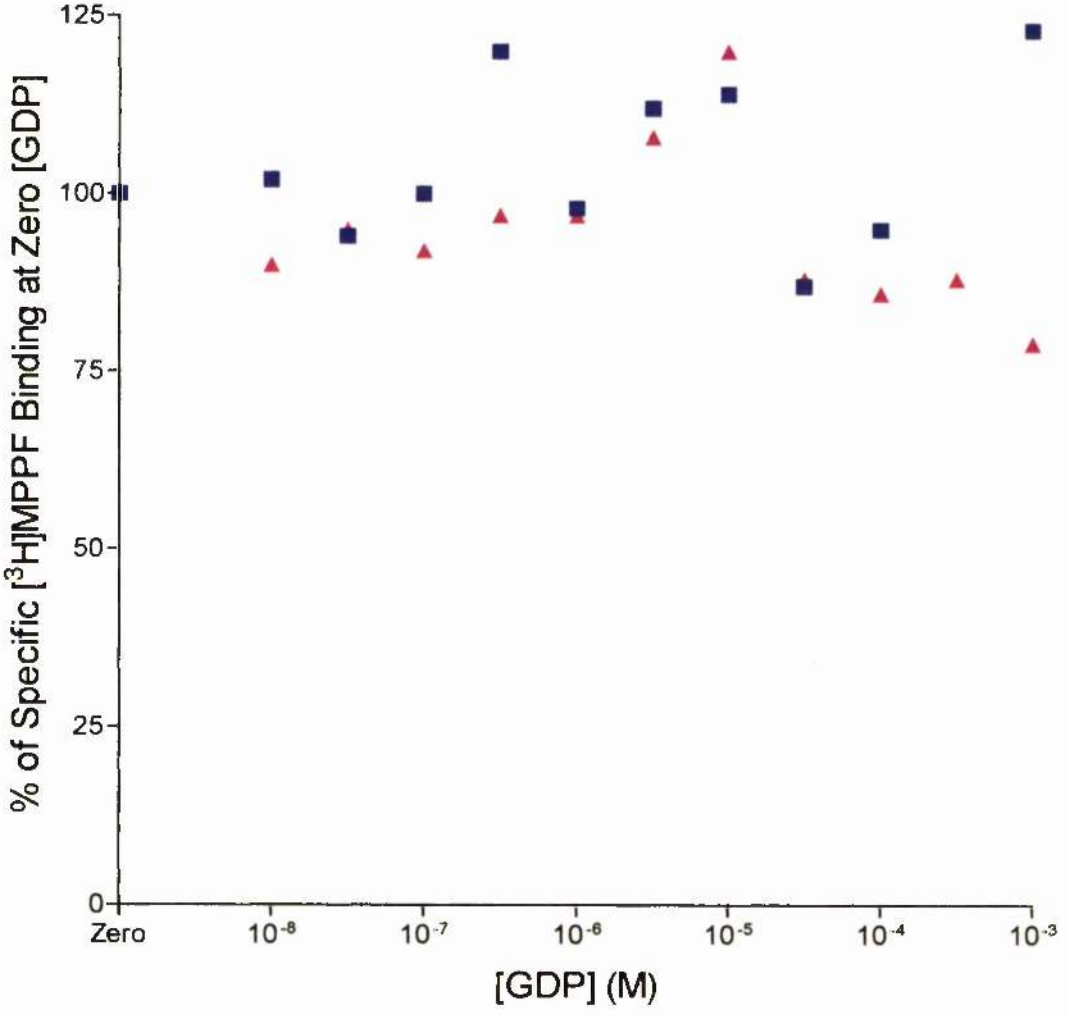
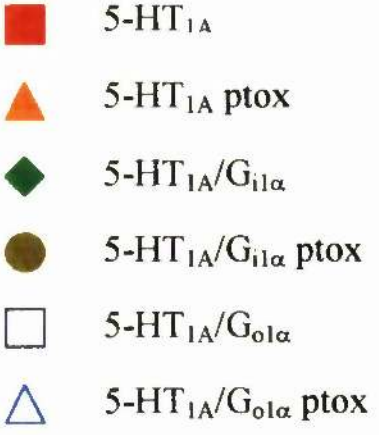

% of Specific [3H]MPPF Binding at Zero [GDP]
125
100
75
50
25
0
Zero
10-8
10-7
10-6
10-5
10-4
10-3
[GDP] (M)

Figure 4.6 **Inhibition of agonist radioligand binding by suramin to membranes prepared from HEK293 cells stably expressing the 5-HT_{1A} receptor and the 5-HT_{1A} receptor-$G_{i1\alpha}$ and $G_{o1\alpha}$ protein fusions**

Percentage of specific [^{3}H]-8-OH-DPAT binding in the absence (100%) or presence of increasing concentrations of suramin in HEK293 cells stably expressing the 5-HT_{1A} receptor and the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins. Pre-treatment with pertussis toxin and increasing concentrations of suramin result in reduced binding of the agonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.



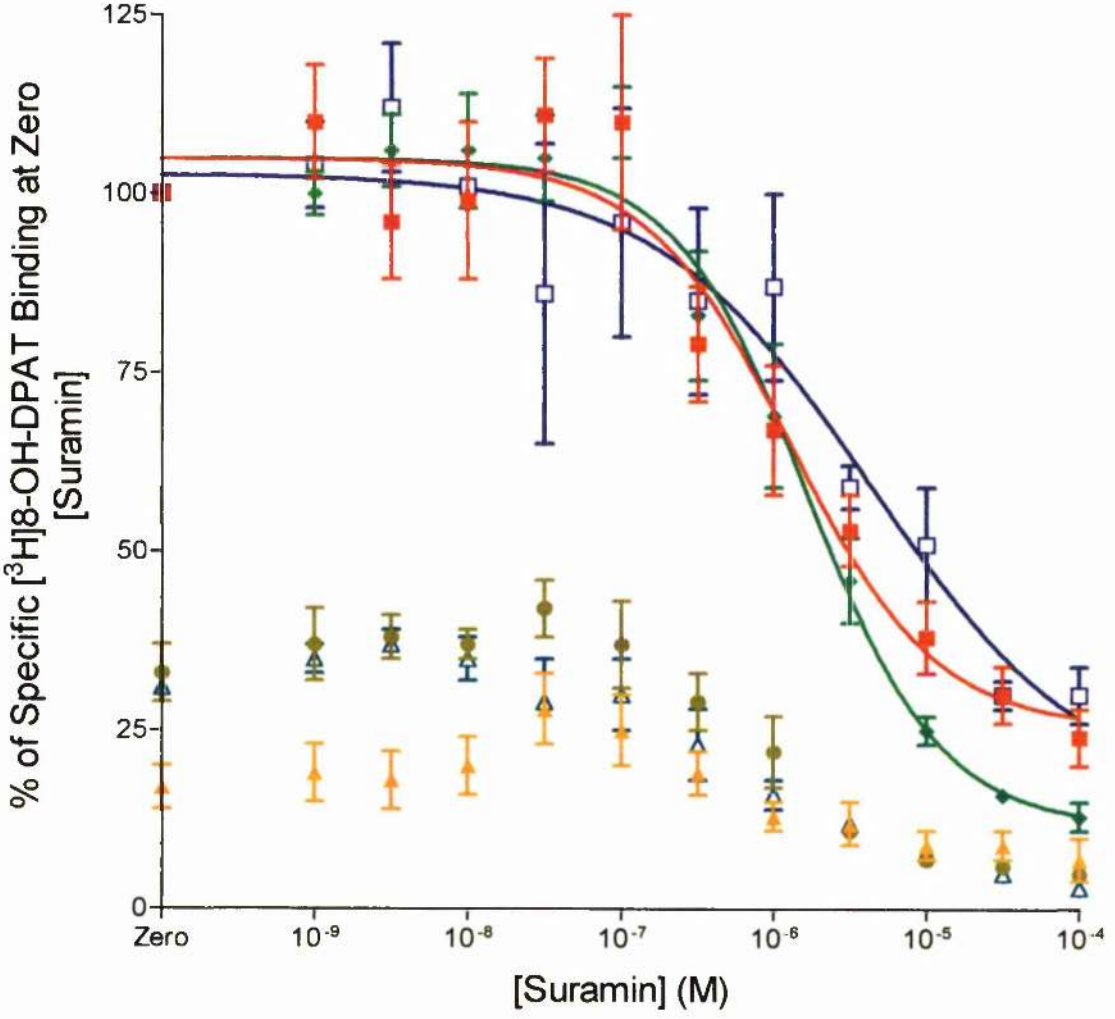

% of Specific [3H]8-OH-DPAT Binding at Zero [Suramin]
125
100
75
50
25
0
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Suramin] (M)

Figure 4.7 **Inhibition of agonist radioligand binding by suramin to membranes prepared from HEK293 cells stably expressing the pertussis toxin resistant 5-HT_{1A}/$G_{i1\alpha}C^{351}$G/I fusion proteins**

Percentage of specific [^{3}H]-8-OH-DPAT binding in the absence (100%) or presence of increasing concentrations of suramin in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{i1\alpha}$ protein fusions with Gly^{351} and Ile^{351} mutations. Increasing concentrations of suramin result in reduced binding of the agonist radioligand Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

5-HT_{1A}/$G_{i1\alpha}C^{351}$G

5-HT_{1A}/$G_{i1\alpha}C^{351}$G ptox

5-HT_{1A}/$G_{i1\alpha}C^{351}$I

5-HT_{1A}/$G_{i1\alpha}C^{351}$I ptox

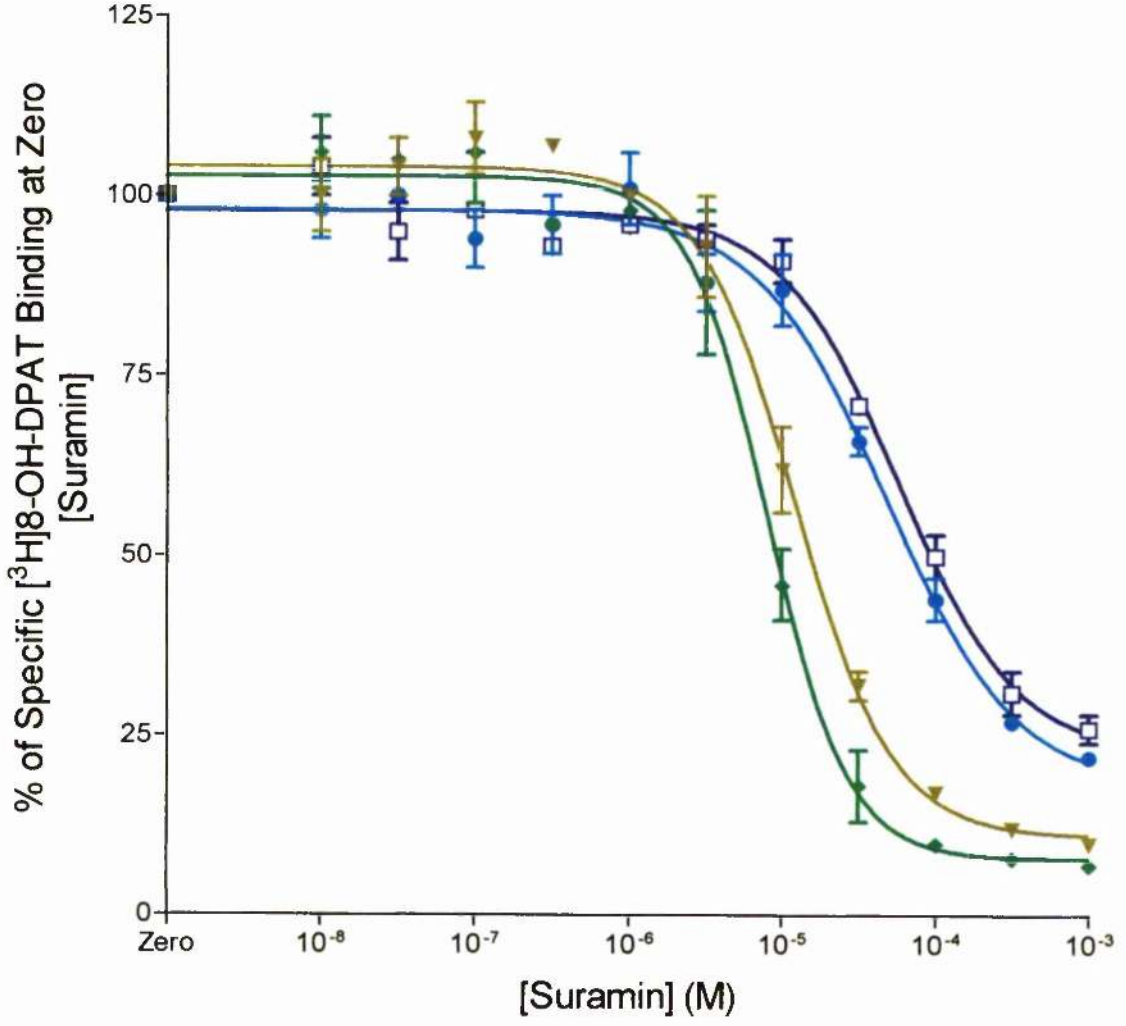

% of Specific [3H]8-OH-DPAT Binding at Zero [Suramin]
125
100
75
50
25
0
Zero
10-8
10-7
10-6
10-5
10-4
10-3
[Suramin] (M)

Table 4.3 **Mean EC_{50} and Hill slope values for suramin prevention of [^{3}H]-8-OH-DPAT binding to membranes prepared from HEK293 cells stably expressing the 5-HT_{1A} receptor and its $G_{i1\alpha}$ fusions proteins**

Table showing the mean +/- S.E.M. EC_{50} and Hill slope values for suramin prevention of specific [^{3}H]-8-OH-DPAT binding to the 5-HT_{1A} receptor, its WT $G_{i1\alpha}$ fusion protein and the pertussis toxin-resistant $G_{i1\alpha}C^{351}G/I$ fusion proteins. Values are means of results from experiments repeated a minimum of three times.

Construct Expressed	EC_{50} (μM) Mean +/-SEM	Hillslope Mean +/- SEM	Number of Replicates
5-HT_{1A}	1.8 +/- 0.8	-1.0 +/- 0.3	4
5-$HT_{1A}/G_{i1\alpha}$	1.7 +/- 0.5	-1.2 +/- 0.1	4
5-$HT_{1A}/G_{i1\alpha}C^{351}G$	1.2 +/- 0.2	-1.4 +/- 0.3	3
5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox	0.8 +/- 0.1	-1.8 +/- 0.3	3
5-$HT_{1A}/G_{i1\alpha}C^{351}I$	4.8 +/- 0.8	-1.1 +/- 0.1	3
5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox	5.8 +/- 0.4	-1.10 +/- 0.04	3

Figure 4.8 **Inhibition of agonist radioligand binding by suramin to membranes prepared from HEK293 cells stably expressing the pertussis toxin resistant 5-HT_{1A}/$G_{o1\alpha}C^{351}$G/I fusion proteins**

Percentage of specific [^{3}H]-8-OH-DPAT binding in the absence (100%) or presence of increasing concentrations of suramin in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{o1\alpha}$ protein fusions with Gly^{351} and Ile^{351} mutations. Increasing concentrations of suramin result in reduced binding of the agonist radioligand Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

- ▼ 5-HT_{1A}/$G_{o1\alpha}C^{351}$G
- ◆ 5-HT_{1A}/$G_{o1\alpha}C^{351}$G ptox
- ● 5-HT_{1A}/$G_{o1\alpha}C^{351}$I
- □ 5-HT_{1A}/$G_{o1\alpha}C^{351}$I ptox

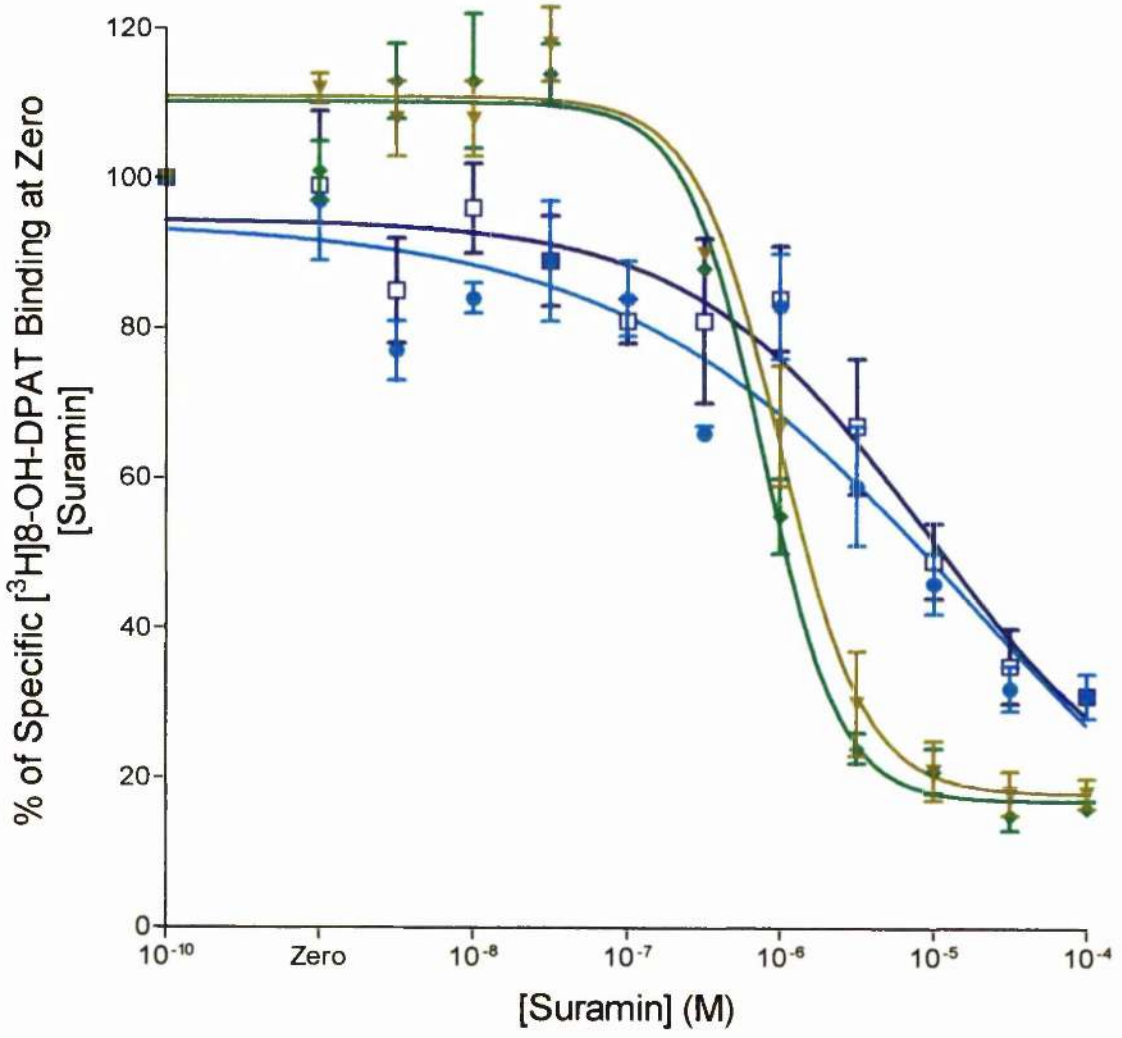

% of Specific [3H]8-OH-DPAT Binding at Zero [Suramin]
120
100
80
60
40
20
0
10-10
Zero
10-8
10-7
10-6
10-5
10-4
[Suramin] (M)

Table 4.4 **Mean EC_{50} and Hill slope values for suramin displacement of [^{3}H]-8-OH-DPAT binding to membranes prepared from HEK293 cells stably expressing the 5-HT_{1A} receptor and its $G_{o1\alpha}$ fusions proteins**

Table showing the mean +/- S.E.M. EC_{50} and Hill slope values for suramin affect on specific [^{3}H]-8-OH-DPAT to the 5-HT_{1A} receptor, its WT $G_{o1\alpha}$ fusion protein and the pertussis toxin-resistant $G_{o1\alpha}C^{351}G/I$ fusion proteins. Values are means of results from experiments repeated a minimum of three times.

Construct Expressed	EC_{50} (μM) Mean +/-SEM	Hillslope Mean +/- SEM	Number of Replicates
5-HT_{1A}	1.8 +/- 0.8	-1.0 +/- 0.3	4
5-$HT_{1A}/G_{o1\alpha}$	1.3 +/- 0.6	-1.0 +/- 0.8	3
5-$HT_{1A}/G_{o1\alpha}C^{351}G$	1.2 +/- 0.3	-1.6 +/- 0.2	4
5-$HT_{1A}/G_{o1\alpha}C^{351}G$ ptox	0.8 +/- 0.1	-1.7 +/- 0.2	4
5-$HT_{1A}/G_{o1\alpha}C^{351}I$	6.9 +/- 1.8	-1.1 +/- 0.6	3
5-$HT_{1A}/G_{o1\alpha}C^{351}I$ ptox	6.1 +/- 0.7	-1.0 +/- 0.3	3

Figure 4.9 **Lack of inhibition of antagonist radioligand binding by suramin to membranes prepared from HEK293 cells stably expressing the 5-HT_{1A} receptor**

Percentage of specific [^{3}H]-MPPF binding in the absence (100%) or presence of increasing concentrations of suramin in HEK293 cells stably expressing 5-HT_{1A} receptor. Neither pertussis toxin-treatment, nor increasing concentrations of suramin alter binding of the antagonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

5-HT_{1A}

5-HT_{1A} ptox

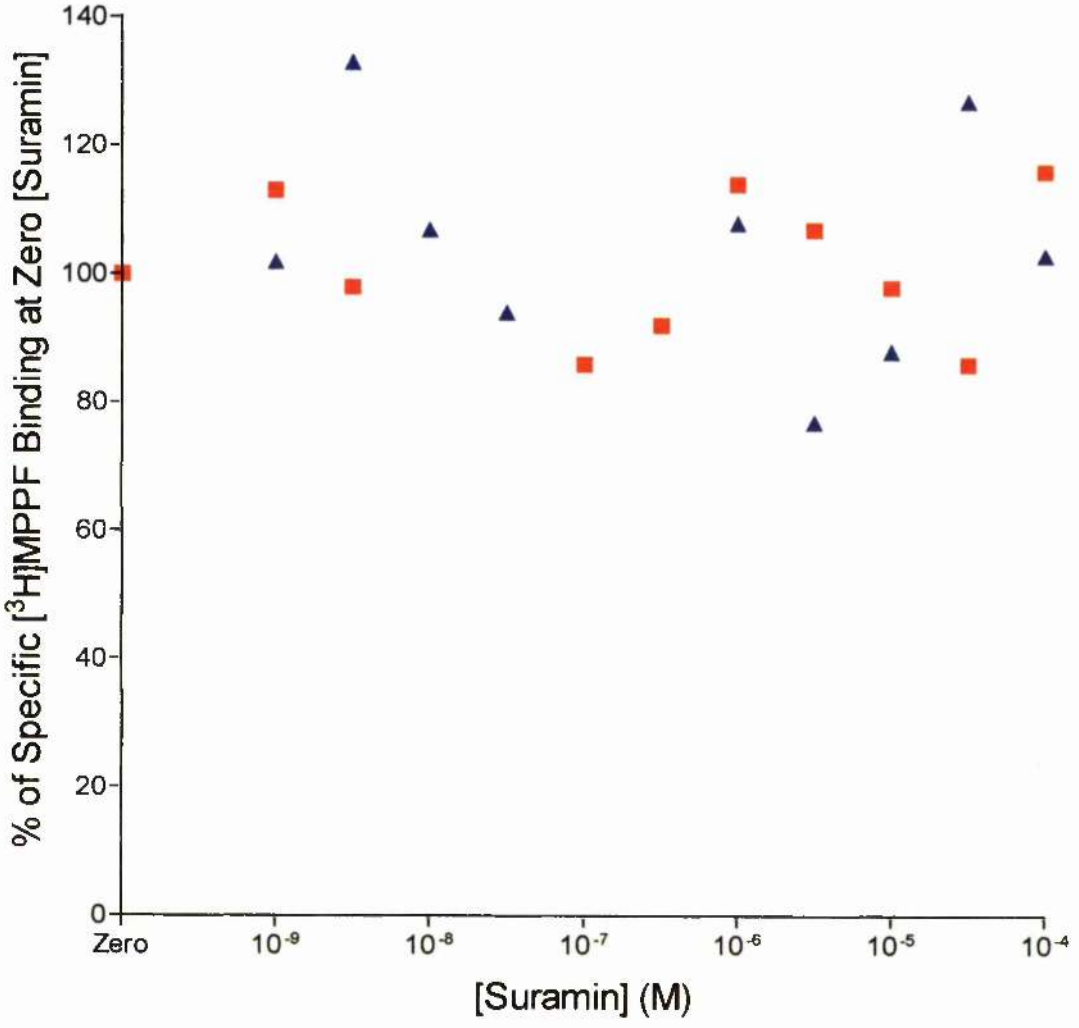

% of Specific [3H]MPPF Binding at Zero [Suramin]
140
120
100
80
60
40
20
0
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Suramin] (M)

Figure 4.10 **Lack of inhibition of antagonist radioligand binding by suramin to membranes prepared from HEK293 cells stably expressing the pertussis toxin-resistant 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion protein**

Percentage of specific [^{3}H]-MPPF binding in the absence (100%) or presence of increasing concentrations of suramin in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{o1\alpha}C^{351}I$ protein fusion. Neither pertussis toxin-treatment, nor increasing concentrations of suramin alter binding of the antagonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

5-HT_{1A}/$G_{o1\alpha}C^{351}I$

5-HT_{1A}/$G_{o1\alpha}C^{351}I$ ptox

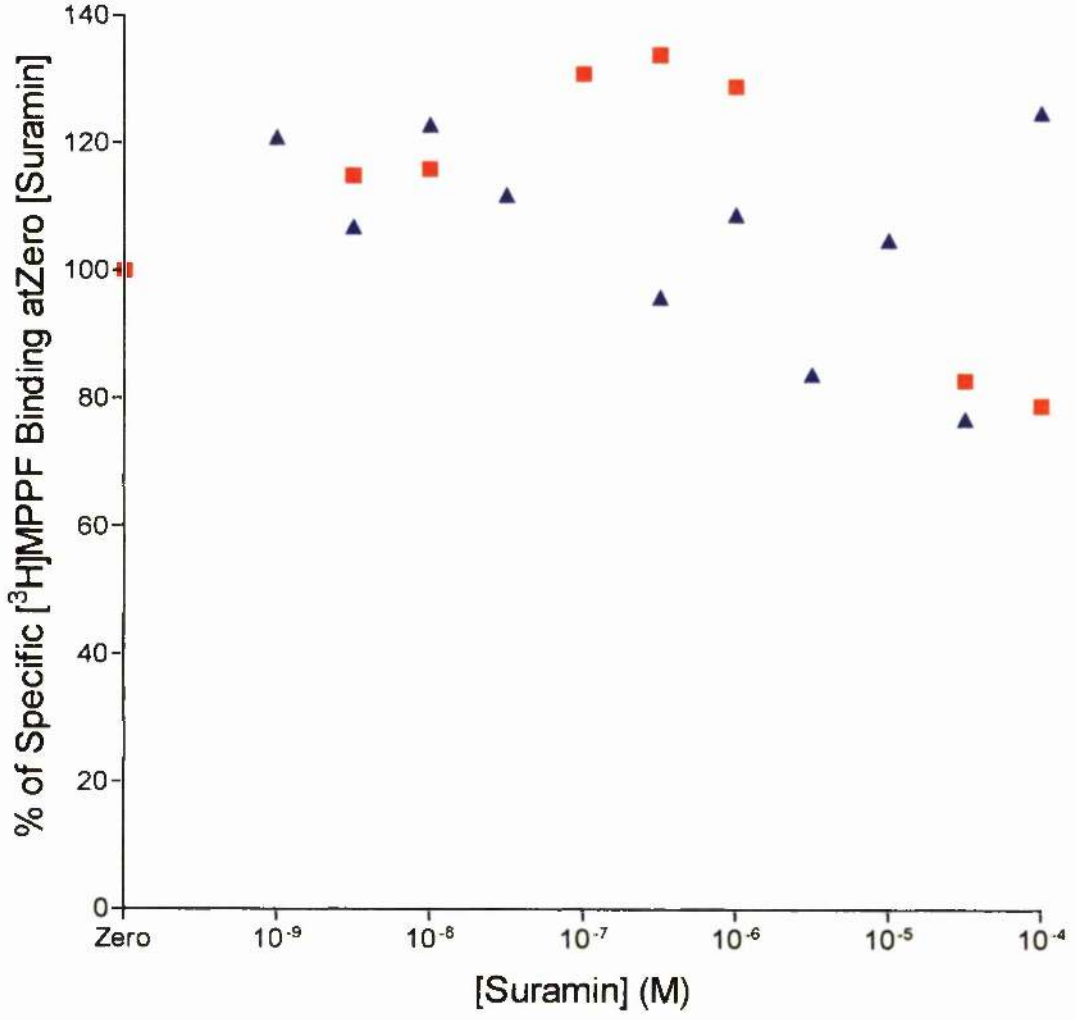

% of Specific [3H]MPPF Binding atZero [Suramin]
140
120
100
80
60
40
20
0
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Suramin] (M)

Figure 4.11 **Competitive biphasic inhibition of antagonist radioligand binding by the agonist 8-OH-DPAT to membranes prepared from HEK293 cells stably expressing the pertussis toxin resistant 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ fusion protein**

Percentage of specific [^{3}H]-MPPF binding in the absence (100%) or presence of increasing concentrations of 8-OH-DPAT in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{i1\alpha}$ protein fusion with the Gly^{351} mutation. Increasing concentrations of 8-OH-DPAT result in the biphasic competition for the antagonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

5-$HT_{1A}/G_{i1\alpha}C^{351}G$

5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox

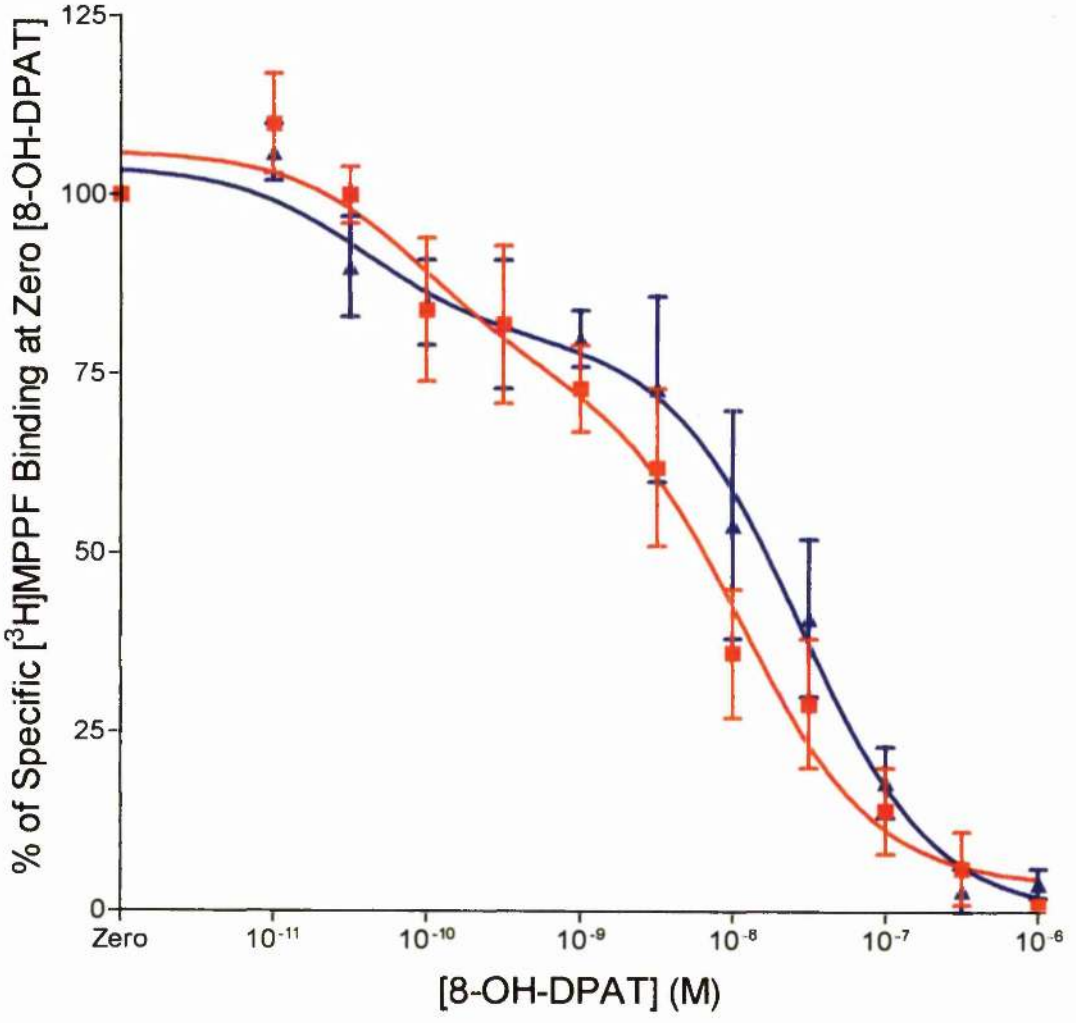

% of Specific [3H]MPPF Binding at Zero [8-OH-DPAT]
125
100
75
50
25
0
Zero
10-11
10-10
10-9
10-8
10-7
10-6
[8-OH-DPAT] (M)

Figure 4.12 **Competitive biphasic inhibition of antagonist radioligand binding by the agonist 8-OH-DPAT to membranes prepared from HEK293 cells stably expressing the pertussis toxin resistant 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein**

Percentage of specific [^{3}H]-MPPF binding in the absence (100%) or presence of increasing concentrations of 8-OH-DPAT in HEK293 cells stably expressing 5-HT_{1A} receptor-$G_{i1\alpha}$ protein fusion with the Ile^{351} mutation. Increasing concentrations of 8-OH-DPAT result in the biphasic competition for the antagonist radioligand. Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

5-$HT_{1A}/G_{i1\alpha}C^{351}I$

5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox

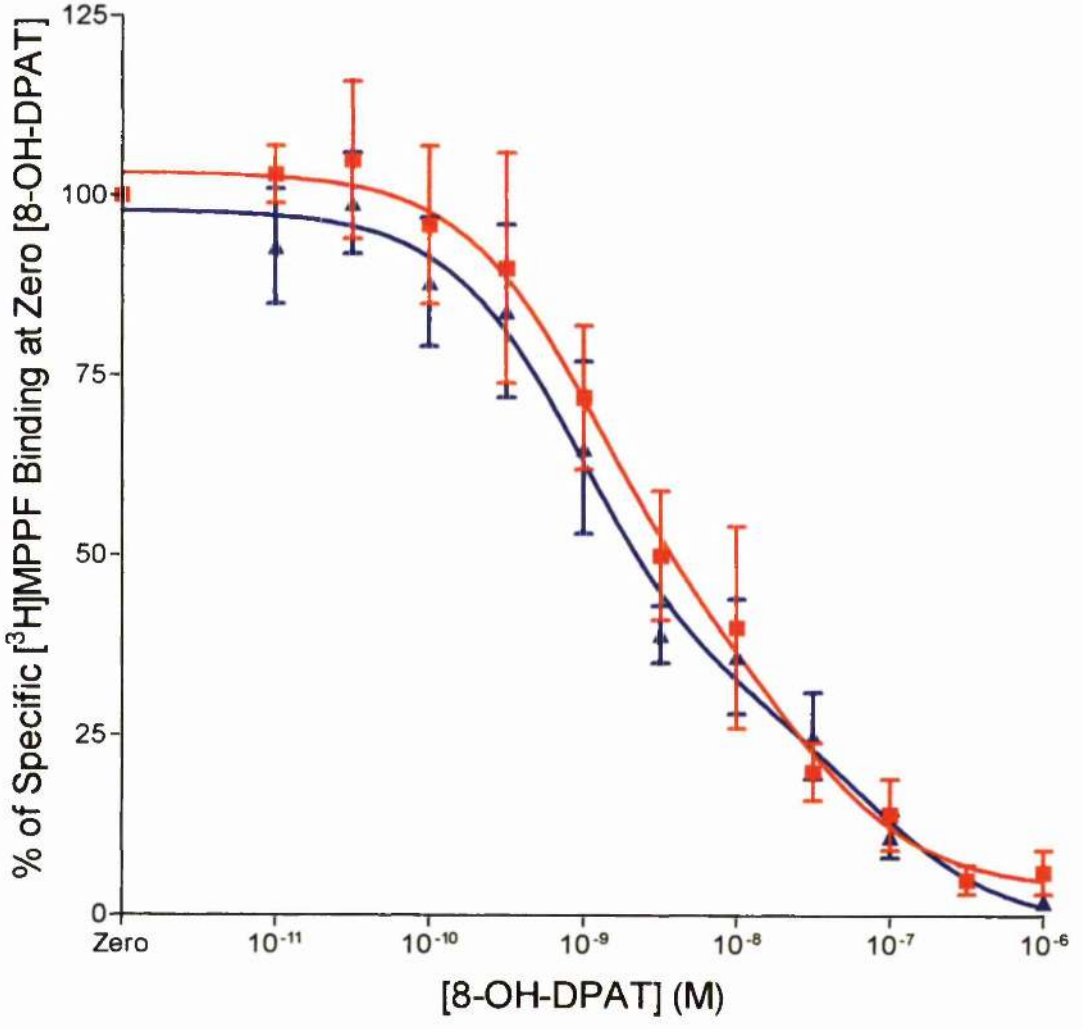

% of Specific [3H]MPPF Binding at Zero [8-OH-DPAT]
125
100
75
50
25
0
Zero
10-11
10-10
10-9
10-8
10-7
10-6
[8-OH-DPAT] (M)

Table 4.5 **Mean high and low affinity EC_{50} and % R_H and R_L values for 8-OH-DPAT displacement of [^{3}H]-MPPF binding to membranes prepared from HEK293 cells stably expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}G/I$ fusion proteins**

Table showing the mean +/- SEM of high and low affinity EC_{50} and $\%R_H$ and R_L values for biphasic 8-OH-DPAT displacement of specific [^{3}H]-MPPF binding to the 5-$HT_{1A}/G_{i1\alpha}C^{351}$G/I fusion proteins. Values are means of results from experiments repeated a minimum of three times.

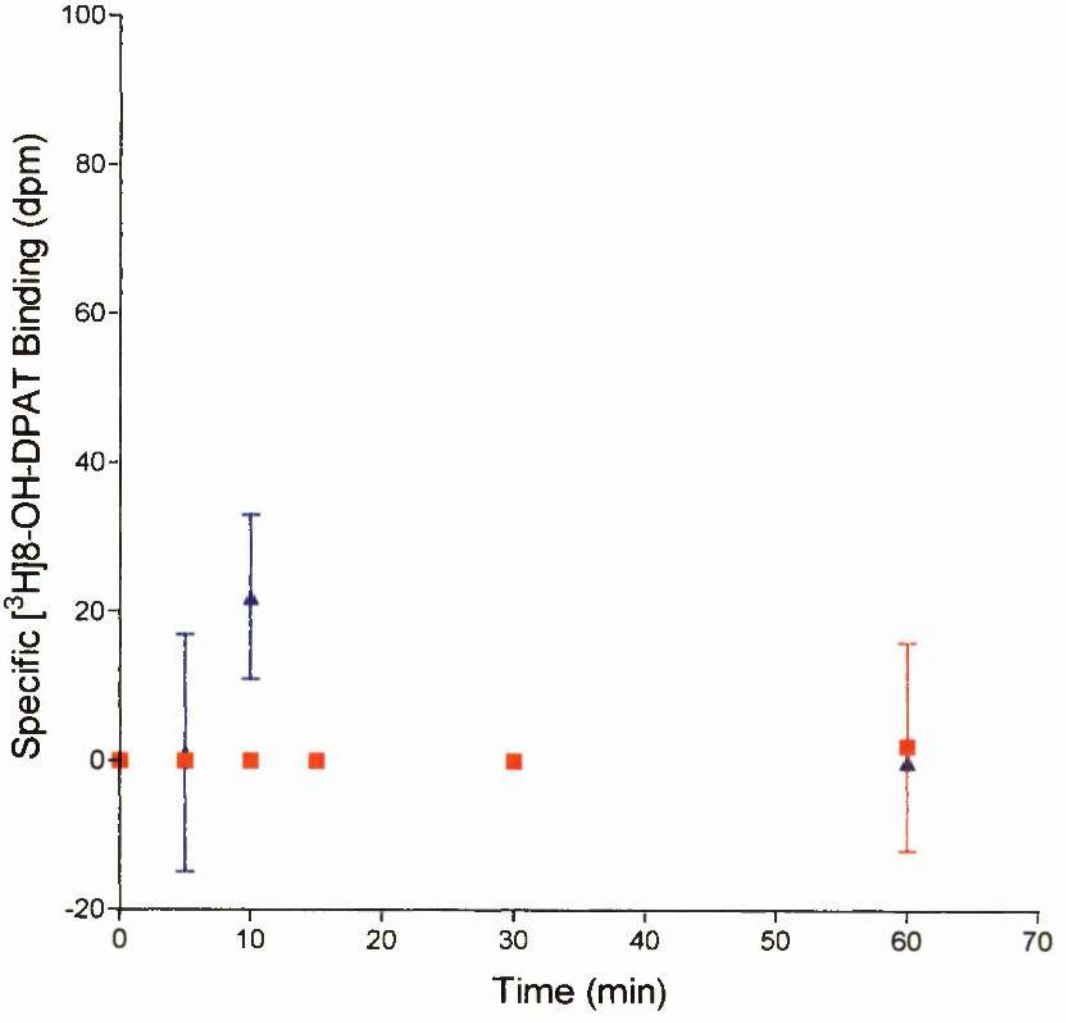
Construct Expressed	**% R_H**	**% R_L**	**EC_{50} 1 (nM)**	**EC_{50} 2 (nM)**
5-HT_{1A}/$G_{i1\alpha}C^{351}G$	30.9+/-0.1	69.1+/-0.2	0.2 +/- 0.1	44.4 +/- 31.3
5-HT_{1A}/$G_{i1\alpha}C^{351}G$ ptox	25.1+/-6.1	74.9+/-7.2	0.1 +/- 0.05	22.2 +/- 9.0
5-HT_{1A}/$G_{i1\alpha}C^{351}I$	54.0+/-18.9	46.0+/-8.4	0.1 +/- 0.08	3.5 +/- 0.7
5-HT_{1A}/$G_{i1\alpha}C^{351}I$ ptox	66.6+/-3.2	33.4+/-2.5	0.9 +/- 0.5	65.0 +/- 43.8

Figure 4.13 **Association binding of the agonist [^{3}H]-8-OH-DPAT to membranes prepared from HEK293 cells mock transfected with the pcDNA3 vector**

Lack of specific binding of [^{3}H]-8-OH-DAPT measured over a 60 minutes incubation period in HEK293 cells mock transfected with the pcDNA3 vector. Samples were filtered and binding halted at 5, 10, 15, 30 and 60 minutes Points are mean+/-S.D. of triplicate determinations and each curve is a representative experiment repeated a minimum of three times.

HEK293

HEK293 ptox


Specific [3H]8-OH-DPAT Binding (dpm)
100
80
60
40
20
0
-20
0
10
20
30
40
50
60
70
Time (min)

4.3 Discussion

Receptors have at least two affinity states for agonists. The high affinity state occurs when the receptor is coupled to a G protein, the low affinity state when the G protein is uncoupled. Receptors that are not G protein coupled can often no longer be labelled with high affinity by agonist radioligands (Emerit *et al.*, 1990). Antagonist binding is independent of affinity state, with binding equal to either G protein-coupled or uncoupled states (Kobilka, 1992). Activated receptor, or agonist bound receptor, decreases the affinity of the G_α subunit of the G protein for GDP, promoting its release. Conversely, an excess of GDP, or non-hydrolysable analogues of GTP such as Gpp(NH)p, lowers the affinity of the G protein for the receptor (Hepler and Gilman 1992). The difference in affinity of a compound for these states can give an indication of its intrinsic activity (Birdsall and Lazareno, 1997).

It had been previously found using [^{35}S]-GTPγS binding studies that the more hydrophobic the amino acid at position 351 in the $G_{i1\alpha}$ subunit, the greater the affinity the α_{2A}-adrenoceptor had for G protein binding (Bahia *et al.*, 1998). Mutation of Cys^{351} to each of the other nineteen naturally occurring amino acids demonstrated that the Ile^{351} mutation produced the greatest UK-14304 stimulated binding of [^{35}S]-GTPγS to the $G_{i1\alpha}$ protein (Bahia *et al*, 1998), one and a half times greater than binding to the WT Cys^{351} protein. However, Waldhoer *et al* (1999), found that in fusion proteins between the A_1 adenosine receptor and the $G_{i1\alpha}$ G protein subunit, mutation of Cys^{351} to either Ile^{351} or Gly^{351} decreased the affinity of the receptor for the G protein. Given these contrasting results, it was important to examine the effects of these mutations on the affinity of the 5-HT_{1A} receptor for the fused $G_{i1\alpha}$ or $G_{o1\alpha}$ G proteins.

To approach this, GDP prevention of [^{3}H]-8-OH-DPAT binding was used to measure the affinity of the 5-HT_{1A} receptor for fused $G_{\alpha i/o}$ subunits with WT Cys^{351}, or Gly^{351} and Ile^{351} mutations. Following prior treatment with pertussis toxin, to ensure coupling of the receptor to only the fused G_α protein and not endogenous pools of inhibitory G proteins, cells were harvested and membranes prepared. It was found that higher concentrations of GDP were required to displace binding of the agonist radioligand to the fusion between the 5-HT_{1A} receptor and the $G_{i1\alpha}$ G protein with Ile^{351} (EC_{50} 6.9 +/- 2.9μM), than with Gly^{351} (EC_{50} 0.14 +/- 0.05μM) (Table 4.1). Although, pertussis toxin-treatment of cells stably expressing the WT Cys^{351}

fusion was not possible, previous data have indicated that 5-HT_{1A} receptor-G protein fusions couple only to the fused G_{α} subunits and not endogenous G proteins. Given this, the EC_{50} of the Cys^{351} fusion (2.2 +/- 0.5μM) for GDP displacement of agonist radioligand binding, may be a result of coupling to the fused $G_{i1\alpha}$ protein only. These results were supported by previous data published on 5-HT_{1A} receptor-$G_{i1\alpha}$ protein fusions, that showed in an intact cell adenylyl cyclase assay lower concentrations of 5-HT were required to inhibit forskolin-stimulated adenylyl cyclase activity for the Ile^{351} fusion than for the Cys^{351} fusion, followed by the Gly^{351} fusion (Kellett *et al.*, 1999). These data together may indicate a rank order of affinity of G protein for receptor, highest to lowest, of Ile^{351}, followed by Cys^{351} and lastly Gly^{351} for the 5-HT_{1A} receptor.

The results for the $G_{o1\alpha}$ fusion proteins were similar in pattern, with the Ile^{351} mutant requiring a concentration of GDP to displace 50% of agonist radioligand binding of 2.6 +/- 0.6μM (EC_{50}), and the Gly^{351} mutant requiring only 0.1 +/- 0.04μM (EC_{50}) (Table 4.2). This follows the same order of affinity for the $G_{o1\alpha}$ stable cell lines in the intact cell adenylyl cyclase assay in chapter 3 and established for the $G_{i1\alpha}$ fusion proteins in both whole cell adenylyl cyclase and GDP prevention bindings. However, the EC_{50} for GDP prevention of [^{3}H]-8-OH-DPAT binding calculated for the 5-HT_{1A}/$G_{o1\alpha}$ fusion was higher than that of the Ile^{351} mutant (5.9 +/- 3.8μM) but not significantly different. This result may be a reflection of the large standard error of the mean for the 5-HT_{1A} receptor - WT $G_{o1\alpha}$ protein fusion.

Comparison of the results for the 5-HT_{1A} receptor-$G_{i1\alpha}$ and $G_{o1\alpha}$ protein fusions indicates that for corresponding mutations, a higher concentration of GDP is required to produce 50% prevention of [^{3}H]-8-OH-DAPT binding to the $G_{i1\alpha}$ fusion proteins than the $G_{o1\alpha}$ fusion proteins (Tables 4.1 and 4.2). This is consistent with the results from the whole cell adenylyl cyclase assay and previously published data indicating a higher affinity of the 5-HT_{1A} receptor for $G_{i1\alpha}$ coupling than $G_{o1\alpha}$ coupling (Clawges *et al.*, 1997; Bertin *et al.*, 1992; Garnovskaya *et al.*, 1997; Raymond *et al.*, 1993).

A second approach used to examine the affinity of the different G proteins for the 5-HT_{1A} receptor required the use of suramin. Suramin acts as a G protein antagonist with two distinct mechanisms of action. Firstly, at lower than μM concentrations, suramin suppresses the rate of GDP release from purified G protein α

subunits (Freissmuth *et al.*, 1996). This effect is reversed in the presence of a GPCR giving an indication that the binding of suramin and an effector are mutually exclusive (Freissmuth *et al.*, 1996). Secondly, suramin and its analogues disrupt the formation of the ternary complex, i.e. the ligand-receptor-G protein complex, by binding to the site of the G protein-receptor interface (Huang *et al.*, 1990; Hohenegger *et al.*, 1998; Beindl *et al.*, 1996). The ability of suramin to disrupt the formation of this complex depends on the constituents involved. The higher the coupling affinity of the receptor and G protein, the less the ability of suramin to uncouple the complex formation (Waldhoer *et al.*, 1999). Small structural changes in suramin change its affinity for receptors, e.g. suramin is ten fold more potent than NF037 (di-demethylated suramin) at uncoupling the A_1 adenosine receptor, but the two analogues are equipotent at uncoupling the D_2 dopamine receptor (Beindl *et al.*, 1996). Given that these two receptors were shown to interact with identical G_α subunits (Waldhoer *et al.*, 1999), the contact formed between the receptor and G protein is diverse enough to allow selective coupling.

Suramin, ranging from 10nM up to 1mM, produced concentration dependent decreases in [^{3}H]-8-OH-DPAT binding to the 5-HT_{1A} receptor and its various G_α protein constructs. Pertussis toxin treatment significantly decreased agonist binding to membranes expressing the 5-HT_{1A} receptor alone and the 5-HT_{1A} receptor fused to the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins, but had no effect on membranes expressing the pertussis toxin resistant receptor-G protein fusion proteins.

As was found in the GDP displacement bindings, the results from pertussis toxin-treated $G_{i1\alpha}$ fusion expressing membranes demonstrated that the concentration of suramin required to produce a 50% reduction in agonist radioligand binding was significantly higher for the Ile^{351} mutant (5.8 +/- 0.4μM) than for the Gly^{351} mutant (0.8 +/- 0.1μM) (P = 0.0002). While the EC_{50} for the WT G protein receptor fusion (1.7 +/- 0.5μM) was not significantly different from the Gly^{351} mutant EC_{50}, it was from the Ile^{351} mutant, and might indicate the same rank order of affinity of the receptor for G protein as in previous results, highest to lowest, $Ile^{351} > Cys^{351} > Gly^{351}$.

The same pattern is observed in the results from the $G_{o1\alpha}$ protein-receptor fusions. The EC_{50} for suramin prevention of [^{3}H]-8-OH-DPAT binding was significantly higher for the Ile^{351} mutant (6.1 +/- 0.7μM) than for the Gly^{351} mutant (0.8 +/- 0.1μM) (P = 0.0003). This was also the case for the non pertussis toxin-

treated results (P = 0.0142) with the EC_{50} for the WT Cys^{351} mutant falling in between (1.3 +/- 0.6μM), although it was not significantly different from the results of the two mutants. When looked at in unison, all of these data indicate that, $G_{i1\alpha}$ or $G_{o1\alpha}$ proteins with the Ile^{351} mutation have a higher affinity for the agonist-occupied 5-HT_{1A} receptor than the WT Cys^{351} receptor-G protein fusion, with both having greater affinity than the Gly^{351} G_{α} subunits.

Another pattern that emerged throughout the binding experiments was that the $G_{i1\alpha}$ proteins appeared to have a higher affinity for the 5-HT_{1A} receptor than their corresponding $G_{o1\alpha}$ fusions did (Table 4.5). Although these differences were not significant (P > 0.05), this conclusion would be supported by previous findings in the literature (Clawges *et al.*, 1997; Bertin *et al.*, 1992; Garnovskaya *et al.*, 1997; Raymond *et al.*, 1993), where the 5-HT_{1A} receptor preferentially coupled to $G_{i\alpha}$ over $G_{o\alpha}$.

Although differences in Hill slope values were observed throughout the ligand binding assays for the various 5-HT_{1A} receptor-G protein fusions, none of these were significant and no firm conclusions may be drawn from the results.

The 5-HT_{1A} selective antagonist [^{3}H]-MPPF was also examined in GDP and suramin prevention bindings. As antagonists do not distinguish between G protein-coupled and uncoupled affinity states of a receptor, binding to both equally (Kobilka *et al.*, 1992), neither pertussis toxin-treatment, nor addition of increasing concentrations of GDP or suramin, prevented the binding of the antagonist radioligand.

Two-site competitive binding is recorded in two main situations. The first is where there are two distinct classes of receptors to which the ligand will bind, e.g., the β_1 and β_2 adrenergic receptors. The second occurs when the unlabelled ligand has distinct affinities for the two sites. This second situation occurs in figures 4.11 and 4.12, where the receptors exist in two affinity states for agonist binding (Emeritt *et al.*, 1990). In the G protein uncoupled form the receptors have a low affinity for agonist binding, but once the receptor and G protein interact, the receptor changes conformation and exists in the high affinity form. Activated receptor, or agonist bound receptor, decreases the affinity of the G_{α} subunit of the G protein for GDP, promoting its release. Conversely, an excess of GDP, or non-hydrolysable analogues of GTP such as Gpp(NH)p, lowers the affinity of the G protein for the receptor

(Hepler and Gilman 1992). The difference in affinity of a compound for these states can give an indication of its intrinsic activity (Birdsall and Lazareno, 1997). By looking at agonist displacement of antagonist radioligand binding we can measure the fraction of the receptor existing in the two affinity states along with the EC_{50} of agonist displacement of antagonist values for these two states (Table 4.5). It was found that for the 5-HT_{1A} receptor $G_{i1\alpha}C^{351}G$ fusion, only 25.1 +/- 6.1% of the receptor population existed in the high affinity state ($\%R_H$). A significantly greater fraction of the 5-HT_{1A} receptor $G_{i1\alpha}C^{351}I$ fusion existed in the high affinity state, 66.6 +/- 3.2% (P = 0.0039). These data indicate that with Ile at position^{351} in the $G_{i1\alpha}$ protein, the 5-HT_{1A} receptor has a significantly higher affinity for the G protein than when Gly is present at position^{351}, leading to a greater proportion of the 5-HT_{1A} receptor population existing in the high affinity state for agonist binding. These results also indicated that although the close proximity of the receptor and G protein in the fusion construct may favour the G protein coupled state, the construct does not exclusively exist in this conformation.

In order to assess more fully the possible intrinsic constitutive activity of the Ile^{351} G_α protein mutants, the differences in affinity of Gly^{351}, Cys^{351} and Ile^{351} G_α protein mutants for the 5-HT_{1A} receptor and the differences in affinity of the 5-HT_{1A} receptor for $G_{i1\alpha}$ and $G_{o1\alpha}$ proteins, it was decided to conduct a number of GTPase assays which would measure the rate of GTP hydrolysis by the G_α protein fusions.

Chapter 5

High Affinity GTPase Assays With RGS1 and RGS16 Proteins

5.1 Introduction

Ligand activation of second messenger systems by the 5-HT_{1A} receptor is transduced *via* inhibitory G proteins (Boess and Martin 1994; Saudou and Hen, 1994; Albert *et al.*, 1996). Upon receptor activation the G_α subunit changes conformation, resulting in a decrease in affinity for GDP and an increase in affinity for GTP. Binding of this tri-phosphate leads to dissociation of the α and $\beta\gamma$ subunits allowing stimulation of a variety of second messengers (Hamm, 1998). The period of this signal is determined by the intrinsic GTPase activity of the G_α protein subunit. Hydrolysis of GTP to GDP results in a return to the inactive conformation of the G_α subunit and an increased affinity for the free $\beta\gamma$ dimer, re-association of these transducers terminates second messenger activation (Hamm, 1998; Birnbaumer and Birnbaumer 1995; Helmrich and Hofmann 1996).

It has not been possible to accurately measure the rate of GTP hydrolysis until recently, when fusions between GPCRs and G protein α subunits demonstrated a number of interesting characteristics. In some constructs agonist stimulated signalling was transduced exclusively by the fused G protein (Kellett *et al.*, 1999). Utilising G protein α subunits resistant to pertussis toxin catalysed ADP ribosylation coupled to GPCRs meant that in a GTPase assay ligand stimulated turnover of GTP to GDP was by the fused G_α subunit (Burt *et al.*, 1998). The 1:1 stoichiometry of the GPCR to G protein meant that ligand binding could determine not only the exact expression level of the GPCR but also of the G protein. Using this information the exact turnover of GTP to GDP by a single G protein α subunit could be measured.

It has been previously established that addition of 5-HT to membranes expressing the 5-HT_{1A} receptor and its inhibitory G protein fusions results in a concentration-dependent increase in high affinity GTPase activity (Kellett *et al.*, 1999). In constructs between the 5-HT_{1A} receptor and pertussis toxin resistant G protein α subunits a lack of coupling to the endogenous pool of G_i was demonstrated by the lack of effect of pertussis toxin on both inhibition of adenylyl cyclase activity and GTP turnover in the high affinity GTPase assay. A degree of intrinsic constitutive activity was also measured in the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein illustrated by the ability of spiperone to decrease basal GTPase activity in membranes expressing the Ile^{351} construct (Kellett *et al.*, 1999).

Another factor in determining the degree of activation of second messenger systems is the presence of RGS (Regulators of G protein Signalling) proteins (Hong *et al.*, 1993). This family of proteins, consisting of greater than 20 members, act as GAPs (GTPase activating proteins) for the G_i and G_q classes of G protein α subunits (Berman *et al.*, 1996; Watson *et al.*, 1996; Ingi *et al.*, 1998; Kozasa *et al.*, 1998; Hepler, 1999; De Vries *et al.*, 2000). RGS proteins inhibit GPCR signalling in a number of ways. Firstly, their GAP activity decreases the length of time spent in the active GTP-bound confomation of the G_α subunit (Saitoh *et al.*, 1997; Doupnik *et al.*, 1997). Secondly, the binding of the RGS protein to the G_α subunit can hinder its ability to couple to second messenger generators, preventing signal generation (Hepler *et al.*, 1997). Over expression of GST fused forms of these RGS proteins using a bacterial expression system allows their purification and subsequent characterisation.

Using the high affinity GTPase assay the 5-HT_{1A} receptor and its $G_{i1\alpha}$ and $G_{o1\alpha}$ fusions were further characterised together with the effects of the two available recombinant RGS1 and RGS16 proteins.

5.2 Results

GTPase assay conditions were re-established for the 5-HT_{1A} receptor-G protein fusions stably expressed in HEK293 cell lines following conversion to a 96 well plate format and the use of a Packard Topcounter. Initial experiments examined the high affinity GTPase activity of the 5-$HT_{1A}/G_{i1\alpha}C^{351}$G/I constructs at a variety of protein concentrations and incubation periods (Figures 5.1 and 5.2). No significant difference in either basal or 5-HT (100μM) stimulated GTPase activity was found between the incubation periods of 10, 20, 30 and 40 minutes or at four different protein amounts (10, 5, 2.5 and 1.25μg) for either construct. It was concluded that the conditions observed represented V_0 and not conditions where reactants were limiting. The conditions used for the subsequent GTPase assays were an incubation period of 20 minutes with 2.5μg of protein.

5-HT stimulation of an endogenous receptor in HEK293 cells had shown stimulation of cAMP production in the intact cell adenylyl cyclase assay (Figures 3.11 and 3.12). In order to demonstrate that 5-HT stimulated GTPase activity was entirely due to activation of the stably transfected 5-HT_{1A} receptor-G protein fusions, the 5-HT_{1A} selective agonist 8-OH-DPAT was used in parallel with 5-HT to stimulate high affinity GTPase activity (Figure 5.3). In both untreated and pertussis toxin-treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}$G/I fusion proteins, the activation produced by 8-OH-DPAT was not significantly different to that of 5-HT ($P < 0.05$).

The potency of 5-HT (1nM-100μM) to stimulate high affinity GTPase activity was assessed in the seven different stable cell lines. For the 5-HT_{1A} receptor alone, and the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins, treatment with pertussis toxin abolished agonist stimulated high affinity GTPase activity with the exception of the WT $G_{i1\alpha}$ fusion protein where concentrations above 1μM produced a small stimulation of GTPase activity (Figure 5.4). The pEC_{50} values for the 5-HT_{1A} (7.19+/-0.06nM) receptor and the $G_{i1\alpha}$ and $G_{o1\alpha}$ (6.73+/-0.05nM and 7.51+/-0.22nM respectively) pertussis toxin sensitive fusion proteins were not significantly different from one another ($P > 0.05$) (Tables 5.1 and 5.2).

A concentration-dependent increase in high affinity GTPase activity was also seen with the pertussis toxin resistant 5-$HT_{1A}/G_{i1\alpha}C^{351}$G and 5-$HT_{1A}/G_{i1\alpha}C^{351}$I fusion proteins (Figure 5.5A). The pEC_{50} values for the 5-$HT_{1A}/G_{i1\alpha}C^{351}$G protein in the

absence or presence of pertussis toxin (6.11+/-0.07nM and 5.94+/-0.08nM respectively) were not significantly different from each other ($P > 0.05$), a result that may indicate that the fusion protein is not coupling to endogenous G proteins in this assay (Table 5.1). The same was not true for the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein with pEC_{50} values of 7.23+/-0.09nM and 6.50+/-0.07nM for non- and pertussis toxin-treated membranes respectively ($P < 0.05$). This may indicate coupling to the endogenous pool of inhibitory G proteins. Comparison of the pEC_{50} values for the Gly^{351} mutant and the Ile^{351} mutant proteins established that the two non-pertussis toxin-treated samples were significantly different from one another ($P < 0.001$), as were the pertussis toxin-treated samples ($P < 0.01$). This may be a reflection of the higher affinity that $G_{i1\alpha}$ has for the 5-HT_{1A} receptor with the in the Ile^{351} mutation. The pEC_{50} values for both the Gly^{351} and Ile^{351} $G_{i1\alpha}$ protein mutants were significantly different from the WT Cys^{351} $G_{i1\alpha}$ fusion protein ($P < 0.05$). This result supports the hypothesis that the hydrophobicity of the amino acid at position 351 in the $G_{i1\alpha}$ protein is important in 5-HT_{1A} receptor-$G_{i1\alpha}$ protein coupling.

The results for the $G_{o1\alpha}$ fusion proteins were analysed and did not demonstrate any differences between non-treated and pertussis toxin-treated samples for each protein (Gly^{351}: 6.69+/-0.01nM and 6.88+/-0.07nM; Ile^{351}: 7.46+/-0.03nM and 6.71+/-0.22nM respectively), or any differences between the Gly^{351} and Ile^{351} mutants ($P > 0.05$) (Table 5.2). As with the results for the $G_{i1\alpha}$ fusion proteins, the pEC_{50} value for the WT $G_{o1\alpha}$ fusion protein (7.51+/-0.22) was significantly different from the pEC_{50} values for the Gly^{351} and Ile^{351} mutant $G_{i1\alpha}$ fusion proteins ($P < 0.001$). This result again indicates that the interaction between the 5-HT_{1A} receptor and the $G_{i1\alpha}$ protein with the Gly^{351} mutation may not be as strong as the WT $G_{i1\alpha}$ protein or the Ile^{351} mutant $G_{i1\alpha}$ protein interactions. Comparison of the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion protein results showed no differences in the EC_{50} values for 5-HT stimulated high affinity GTPase activity between the samples ($P > 0.05$).

RGS protein-GST fusions had previously been generated in the lab for both RGS1 and RGS16. cDNA encoding these fusion proteins was transformed into competent BL21-S1 bacteria which were grown in 500ml cultures to an OD^{600} of 0.3-0.4 before induction with I.P.T.G. and the expressed protein subsequently purified following four hours growth as per section 2.3. Figure 5.6 shows a representative Coomassie Blue stained gel of the various samples taken during the

purification process. Induction of the RGS-GST protein is denoted by a strong increase in the band present on the gel at approximately 50kDa following addition of IPTG and the purification was followed using Glutathione Sepharose 4B gel. Typically, a protein concentration of between 10 and 20μM was purified using this method.

The effects of these purified RGS proteins were assessed in the high affinity GTPase assay (Figure 5.7, A + B). The agonists 5-HT (100μM) and 8-OH-DAPT (100μM) produced significant increases in GTPase activity over basal for both the 5-$HT_{1A}/G_{i1\alpha}$ Gly^{351} and Ile^{351} pertussis toxin treated membranes ($P < 0.05$). These effects were significantly increased ($P < 0.05$) by the addition of either RGS1 or RGS16 (1μM) to the reaction mixture when compared to agonist alone, indicating functional activity for the purified proteins.

A control experiment was carried out to confirm that the effect of the RGS proteins was due to 5-HT_{1A} receptor activity. Using mock-transfected HEK293 cells, the effects of 5-HT (100μM) and RGS1 (1μM) on GTPase activity were investigated (Figure 5.8). Neither 5-HT, nor RGS1 alone produced any significant increase in GTPase activity over basal. The combination of the two did not have a significant effect either.

The effects of the RGS1 and RGS16 proteins on agonist-activated high affinity GTPase activity was examined at a range of GTP concentrations. In membranes expressing the 5-HT_{1A} receptor alone, over a range of nucleotide concentrations from 25nM to 3μM, 5-HT produced a large increase in V_{max} (1.5 +/- 0.05 fold) without effecting the K_m (253 +/- 21nM and 200 +/- 13nM respectively) for GTP (Figure 5.9, A + B). In the presence of RGS1 at 10μM, 5-HT produced a much more marked increase in high affinity GTPase activity. Along with the three-fold increase in V_{max} there was also an increase in the K_m for GTP (from 256 +/- 52nM to 616 +/- 60nM respectively). Treatment of cells with pertussis toxin prior to harvesting and membrane preparation produced a decrease in V_{max} but no alteration of the K_m for GTP (Figure 5.10, A + B). It also abolished any effects of 5-HT or RGS1 on high affinity GTPase activity.

High affinity GTPase activity in membranes stably expressing the WT 5-HT_{1A} receptor $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins was examined in the presence of RGS1 and 5-HT (Figure 5.11, A + B). 5-HT produced over a four-fold increase in V_{max} for

the $G_{i1\alpha}$ fusion, and a one and a half fold increase for the $G_{o1\alpha}$ fusion, but did not have a significant effect on the K_m for GTP for either construct ($P > 0.05$). Addition of RGS1 (10μM) to the reaction mix produced a significant increase in V_{max} in the presence of 5-HT over 5-HT alone (seven fold for $G_{i1\alpha}$ and 10 fold for $G_{o1\alpha}$) ($P < 0.05$) and in K_m (seven fold for both $G_{i1\alpha}$ and $G_{o1\alpha}$) ($P < 0.05$). Pertussis toxin treatment reduced the V_{max} for both $G_{i1\alpha}$ and $G_{o1\alpha}$ fusions for all treatments with the effects of RGS1 no longer being significant (Figure 5.12, A + B).

The effects of RGS1 and RGS16 were examined in much greater detail with the pertussis toxin-resistant mutants of both $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins. The effect of RGS16 was assessed at seven different concentrations (1nM, 5nM, 10nM, 50nM, 100nM, 500nM, and 1μM) and those of RGS1 at eight different concentrations (as RGS16 plus 5μM). The basal and 5-HT stimulated high affinity GTPase activity for $5\text{-}HT_{1A}/G_{i1\alpha}C^{351}G$ was measured in the presence of the range of RGS protein concentrations. In Figure 5.13, increasing concentrations of RGS1 had no effect on either the V_{max} or K_m for GTP of basal GTPase activity ($P > 0.05$) (Table 5.3). In the presence of 5-HT (100μM)(Figure 5.14), an increase in both V_{max} and K_m are measured with increasing concentrations of RGS1. Stimulation by 5-HT causes a significant increase in V_{max} over basal ($P < 0.05$), but has no effect on the K_m for GTP ($P > 0.05$). The effects of RGS1 on V_{max} become significant at 50nM ($P = 0.0015$), but despite clear increases, those on K_m only become significant once the concentration of RGS1 has reached 1μM ($P = 0.0157$).

In the presence of RGS16 (Figure 5.15), as with RGS1, no increase in either V_{max} or K_m is measured for basal GTPase activity ($P > 0.05$) (Table 5.3). 5-HT alone produces a significant increase in V_{max} over basal (Figure 5.16) ($P = 0.0015$), but has no effect on K_m ($P > 0.05$). Following the addition of RGS16 at increasing concentrations, the effects on V_{max} over 5-HT stimulation become significant at 50nM ($P = 0.0099$) and the effects on K_m at 100nM ($P < 0.05$).

In the presence of increasing concentrations of RGS1, basal $5\text{-}HT_{1A}/G_{i1\alpha}C^{351}I$ GTPase activity increases in terms of V_{max} and K_m (Figures 5.17 and 5.18). The increase in V_{max} becomes significant at 5nM ($P = 0.0235$), but although the increase in K_m is apparent over the eight RGS protein concentrations, increasing from 219 +/- 36nM to 447 +/- 69nM, it is not statistically significant ($P > 0.05$) (Table 5.4). Following stimulation with 5-HT, V_{max} increases significantly over basal ($P =$

0.0025) without a change in K_m. With the addition of RGS1, the V_{max} and K_m become significant over 5-HT stimulation at 100nM (P = 0.0083 and 0.0303 respectively).

The effects of RGS16 are similar, although neither the increases in V_{max}, rising from 38 +/- 10 to 81 +/- 15 (pmol/mg/min) (Table 5.4), nor in K_m, rising from 210 +/- 48nM to 411 +/- 151nM, for basal GTPase activity become significant (P > 0.05) (Figure 5.19). The effects on V_{max} in the presence of 5-HT are significant over basal (P < 0.05), and in the presence of RGS16 become significant over 5-HT stimulation alone at 1nM (P = 0.0455) (Figure 5.20). Stimulation with 5-HT alone has no effect on K_m, but the addition of RGS16 from concentrations of 5nM and above has a significant effect (P = 0.0226).

Comparison of the effects of RGS1 and RGS16 on GTPase activity of the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ construct demonstrated no significant effect on basal GTPase activity for either RGS protein (Figure 5.21, A) and similar effect for both proteins on 5-HT stimulated high affinity GTPase activity (Figure 5.21, B). When this is examined in terms of fold stimulation in GTPase activity over 5-HT stimulation (Figure 5.22), non linear regression predicts a higher maximal increase in activity for RGS1 (27 fold) than for RGS16 (4 fold), although given that the curve may be either reaching a plateau or, the exponential phase of any RGS protein effect, these predictions are unlikely to be accurate. Although the curve fit for RGS1 and RGS16 effects on basal 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ GTP hydrolysis may not be completely accurate, it is apparent that the RGS proteins have a significant effect of basal turnover of GTP (Figure 5.21, A). The RGS protein effects on GTP turnover following 5-HT stimulation are almost identical (Figure 5.21, B), with neither protein having reached a maximal effect at the highest concentration used. When their effects are examined in terms of fold increase in turnover number over 5-HT stimulation, RGS16 appears to have a greater effect (Figure 5.22), but given the large standard error for RGS16 at 1μM, it is not surprising that this difference is not significant.

Basal GTPase activity for the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein is significantly modified by the addition of RGS1 at 1μM in terms of V_{max} (P = 0.0427), but the K_m does not significantly change (Figure 5.23) (Table 5.5). Stimulation of high affinity GTPase activity with 5-HT significantly increases V_{max} over basal (P = 0.0025) but doesn't effect K_m (Figure 5.24). Increases in V_{max} and K_m in the presence of RGS1 both become significant at 50nM (P = 0.0126 and 0.019 respectively). The

effects of RGS16 are similar, but basal V_{max} and K_m are not significantly effected even at 1μM (Figure 5.24) (Table 5.5). 5-HT produces a significant increase in V_{max} over basal (P = 0.0191) and in the presence of RGS16, V_{max} increases significantly over 5-HT stimulation at 5nM (P = 0.0089) (Figure 5.25). 5-HT alone has no effect on the K_m for GTP, but in the presence of RGS16, increases in K_m become significant at 50nM (P = 0.0122).

For the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion, RGS1 produces significant increases in both V_{max} and K_m of basal GTPase activity (Figure 5.27) (Table 5.6). At 10nM the increase in V_{max} is significant (P = 0.0012) over basal and at 100nM the effects on K_m are significant (P = 0.0088). 5-HT produced a significant increase in V_{max} over basal (P = 0.0015), but again did not effect the K_m for GTP (Figure 5.28). In the presence of increasing concentrations of RGS1 there was a significant increase in V_{max} at 1nM (P = 0.008) and in K_m at 50nM (P = 0.0019) over 5-HT stimulation. The second RGS protein, RGS16 produced similar effects on basal GTPase activity (Figure 5.29) (Table 5.6). There was a significant increase in basal GTPase V_{max} at 10nM (P = 0.0164), but no statistical increase in K_m until 1μM (P < 0.05). 5-HT produced a significant increase over basal in V_{max}, but not the K_m for the GTPase activity of the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion (Figure 5.30). For both V_{max} and K_m, the increasing concentrations of RGS16 produced significant increases over 5-HT stimulation alone at 5nM (P = 0.0131 and 0.0184 respectively).

Comparison of the two RGS proteins effects shows that neither had any real effect on basal turnover of GTP (Figure 5.31, A), but both produced a similar significant effect on 5-HT stimulated GTPase activity (Figure 5.31, B). In terms of fold stimulation over 5-HT, RGS1 and RGS16 produce similar increases in stimulation, with RGS1 appearing to produce increases at lower concentrations that RGS16 (Figure 5.32). Comparison of the effects of the two RGS proteins demonstrated similar increases in GTP turnover for both basal and 5-HT stimulation (Figure 5.31, A + B). This is also reflected in the fold stimulation over 5-HT (Figure 5.32), with no significant difference in the increases produced by either RGS1 or RGS16 (P > 0.05).

In order to determine if endogenous RGS protein effects were being lost during membrane preparation the two fractions normally discarded during 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ membrane preparation were assayed for RGS protein activity (Figure

5.33, A). Neither fraction produced an increase in GTPase activity, either basal or stimulated, but actually produced a slight decrease in activity. As a control, purified RGS1 (1μM) was shown to produce increases in high affinity GTPase activity similar to previous observations. Given these results it was decided that to assay these fractions at a range of GTP concentrations would not show any effects on K_m and V_{max}.

Secondly, to investigate further the possible loss of RGS proteins during membrane preparation, cells were treated with 5-HT (100μM) prior to harvesting to see if a stronger interaction with the membrane could be induced by interaction of any endogenous RGS proteins with the activated 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion protein (Figure 5.33, B). There was no significant increase in basal or 5-HT stimulated GTPase activity in these membranes over untreated control membranes. As a control, purified RGS1 was assayed for activity in the presence and absence of 5-HT and produced a response similar to previous experiments. It was decided that no significant effect on either V_{max} or K_m would be seen in an assay of a range of GTP concentrations.

To examine endogenous expression of RGS proteins, quantitative reverse transcriptase PCR was carried out on HEK293 DNA. A list of RGS proteins reportedly expressed either ubiquitously or in the kidney was drawn up (Table 5.1) and primers and probes were designed for RGS2, RGS3, RGS4, RGS9 and GRK2. With the exception of RGS9, cDNA was available for the RGS proteins and was used to set the PCR conditions for the taqman experiments. Figure 5.34, panel A illustrates the successful establishment of PCR conditions for RGS2, showing that for the 50:50nM ratio of reverse to forward primer (yellow/green/red lines) there was a lower level of amplification than with the 300:300nM ratio or the 900:900nM ratio (group of lines to the left). From this experiment, the concentration of forward to reverse primer was set at 300:300nM with the concentration of the probe remaining constant (100nM final). Identical amplification for RGS3 and RGS4 was seen at the three different primer concentrations so the lowest concentration of 50:50nM was selected. For GRK2 (Figure 5.34, B), no discernible amplification was detected at any of the three primer concentrations, and no further experiments were performed on this protein. As no cDNA was available for RGS9, genomic DNA was used as a

template for experiments to establish PCR conditions. As with GRK2, no amplification was detected and no further work was carried out with this protein.

With PCR conditions set, an experiment was performed to assess the expression of RGS proteins in HEK293 cDNA. Standard curves for RGS2, RGS3 and RGS4 were also run using a range of cDNA concentrations (10ng – 0.1pg) and as a control, expression of 18S, a ribosomal DNA was assessed. Expression of 18S is thought to be similar throughout most cell types and is used as a reference to quantify the levels of unknown DNA. For RGS2, RGS3 and RGS4, replication using HEK293 DNA as a template occurred between 30 and 35 cycles. This would normally indicate low levels of expression, but problems occurred with the standard curves with no replication being recorded below 1ng of cDNA. It was speculated that this was due to either impurity of the cDNA samples and the overestimation of their concentration, or due to large amounts of secondary structure in the cDNA.

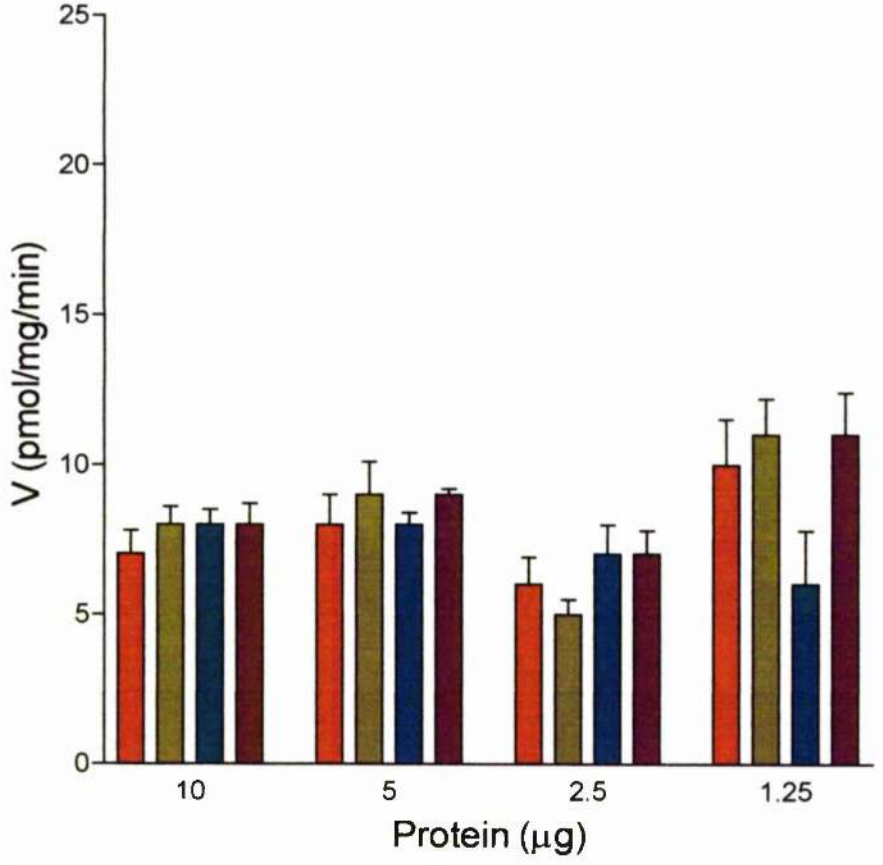
In an attempt to solve these possible problems, the DNA was isopropanol precipitated to remove any impurities and the concentration estimated using OD^{260}. It was found that the concentrations were not significantly different to previous estimations. In an attempt to remove secondary structure, 5% DMSO was added to the reaction mixture and the standard curves carried out again. This did not improve on the results of the previous experiments. No further experiments were performed.

Figure 5.1 **Basal and 5-HT stimulated high affinity GTPase activity in pertussis toxin treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}G$ fusion protein**

Panel A: Basal high affinity GTPase activity in pertussis toxin pre-treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}G$ fusion protein. Panel B: The ability of a previously established maximal 5-HT concentration (Kellett *et al.*, 1999) to stimulate 5-HT_{1A}/$G_{i1\alpha}C^{351}G$ high affinity GTPase activity.

Four different membrane amounts (10, 5, 2.5 and 1.25μg) and three different timepoints (20, 30 and 40min.) were examined to establish appropriate assay conditions. Data are mean+/-SD of triplicate determinations and are representative of at least three similar experiments.

A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15
10
5
0
V (pmol/mg/min)
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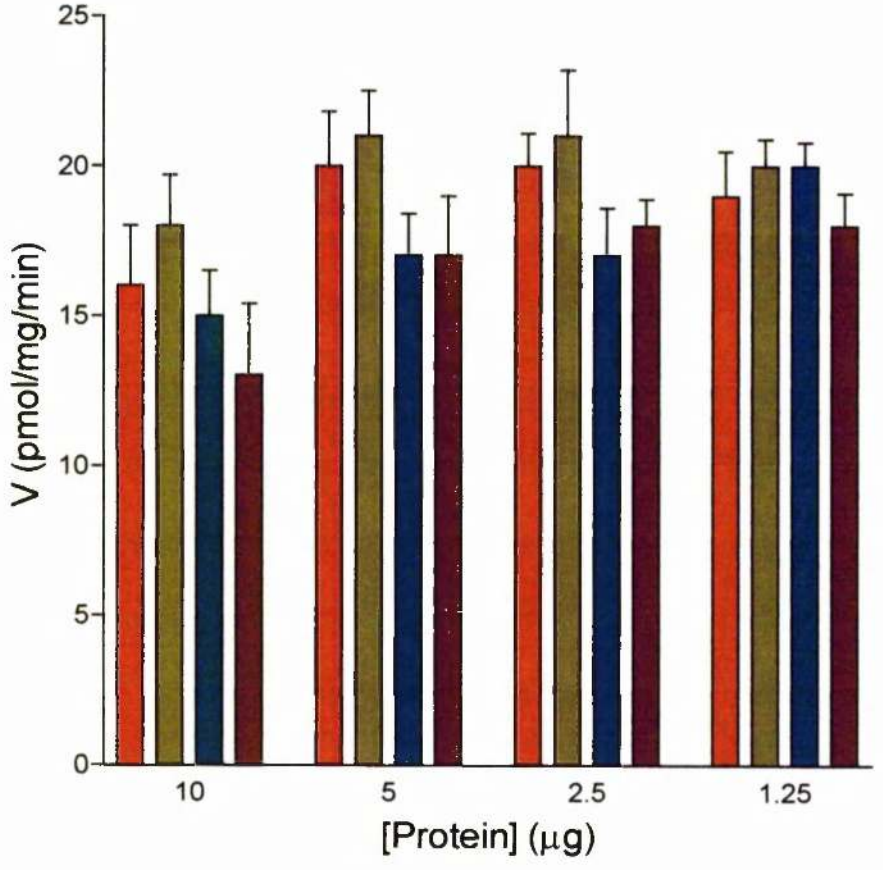
5
2.5
1.25
Protein (μg)

B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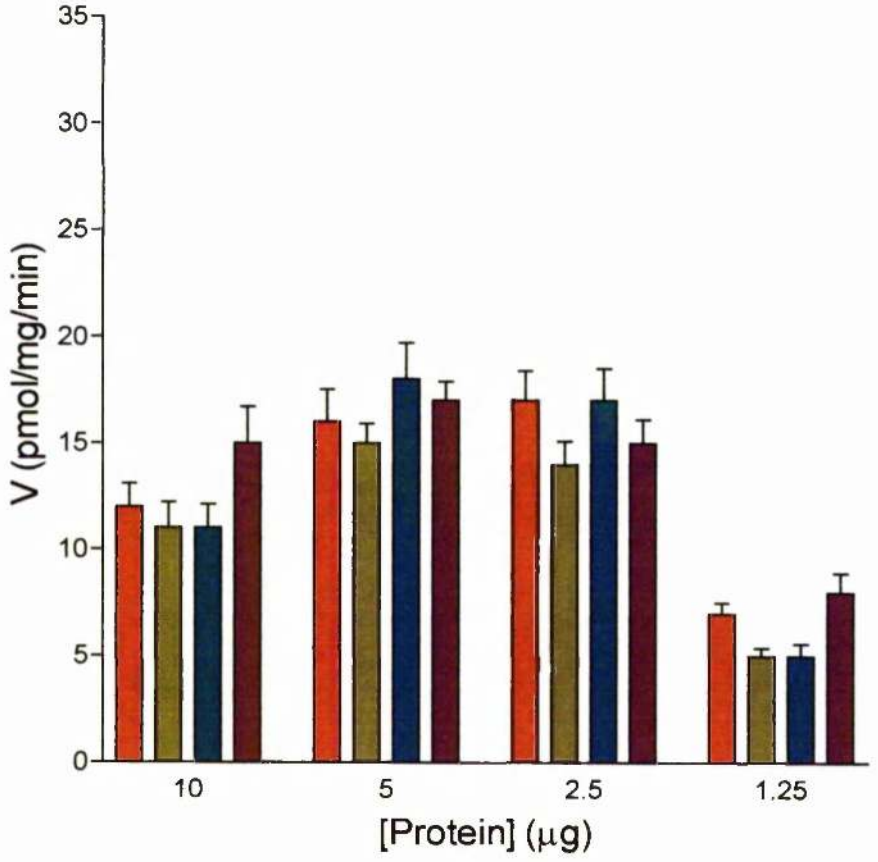
15
10
5
0
V (pmol/mg/min)
10
5
2.5
1.25
[Protein] (μg)

Figure 5.2 **Basal and 5-HT stimulated high affinity GTPase activity in pertussis toxin treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein**

Panel A: Basal high affinity GTPase activity in pertussis toxin pre-treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein. Panel B: The ability of a previously established maximal 5-HT concentration (Kellett *et al.*, 1999) to stimulate 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ high affinity GTPase activity.

Four different membrane amounts (10, 5, 2.5 and 1.25μg) and three different timepoints (20, 30 and 40min.) were examined to establish appropriate assay conditions. Data are mean+/-SD of triplicate determinations and are representative of at least three similar experiments.

A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20
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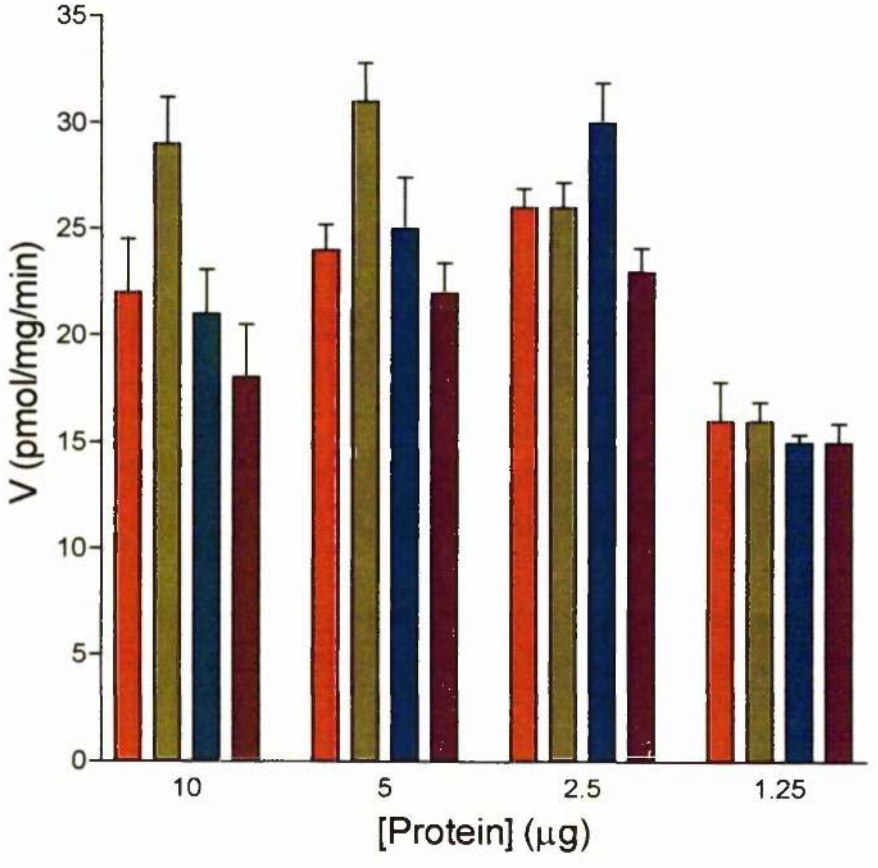
10
5
0
V (pmol/mg/min)
10
5
2.5
1.25
[Protein] (µg)

B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1.25
[Protein] (µg)

Figure 5.3 **Basal and agonist stimulated high affinity GTPase activity in membranes expressing the 5-HT_{1A} receptor fused to Gly^{351} and Ile^{351} $G_{i1\alpha}$ protein mutants**

Basal and agonist stimulated high affinity GTPase activity in non-, and pertussis toxin pre-treated membranes expressing either the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ or the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein. Stimulation with either 5-HT (100μM) or the 5-HT_{1A} selective agonist 8-OH-DAPT (100μM) was not significantly different ($P > 0.05$). Prior treatment of cells prior to harvesting with pertussis toxin did not significantly alter high affinity GTPase activity ($P > 0.05$). Data are means of triplicate determinations and are representative of at least three similar experiments.

- 5-$HT_{1A}/G_{i1\alpha}C^{351}G$
- 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox
- 5-$HT_{1A}/G_{i1\alpha}C^{351}I$
- 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox

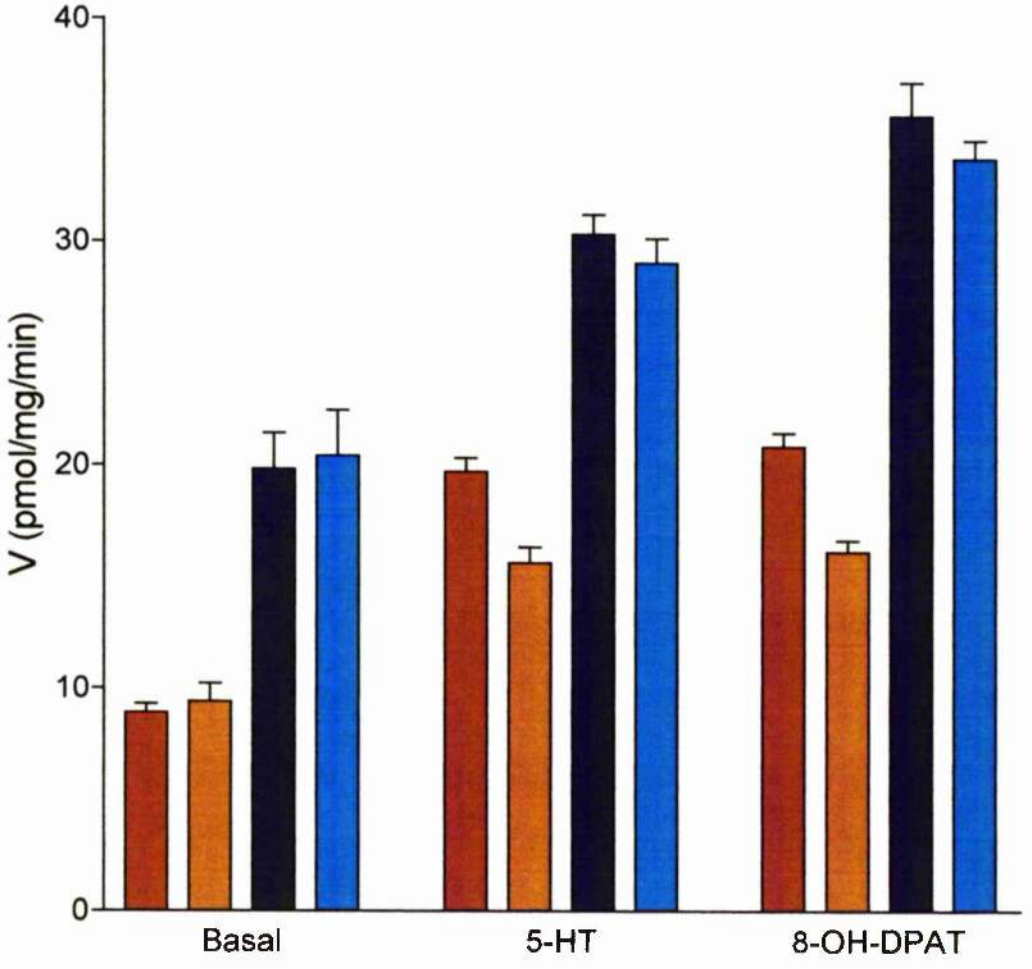

40
30
20
10
0
V (pmol/mg/min)
Basal
5-HT
8-OH-DPAT

Figure 5.4 **5-HT concentration response curves for activation of GTPase activity in the 5-HT_{1A} receptor and its WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins**

The potency of 5-HT to stimulate high affinity GTPase activity in both untreated and pertussis toxin treated membranes expressing the 5-HT_{1A} receptor and its WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins was examined. Data are mean+/-S.D. of triplicate determinations and are representative of at least three similar experiments.

5-HT_{1A}

5-HT_{1A} ptox

5-$HT_{1A}/G_{i1\alpha}$

5-$HT_{1A}/G_{i1\alpha}$ ptox

5-$HT_{1A}/G_{o1\alpha}$

5-$HT_{1A}/G_{o1\alpha}$ ptox

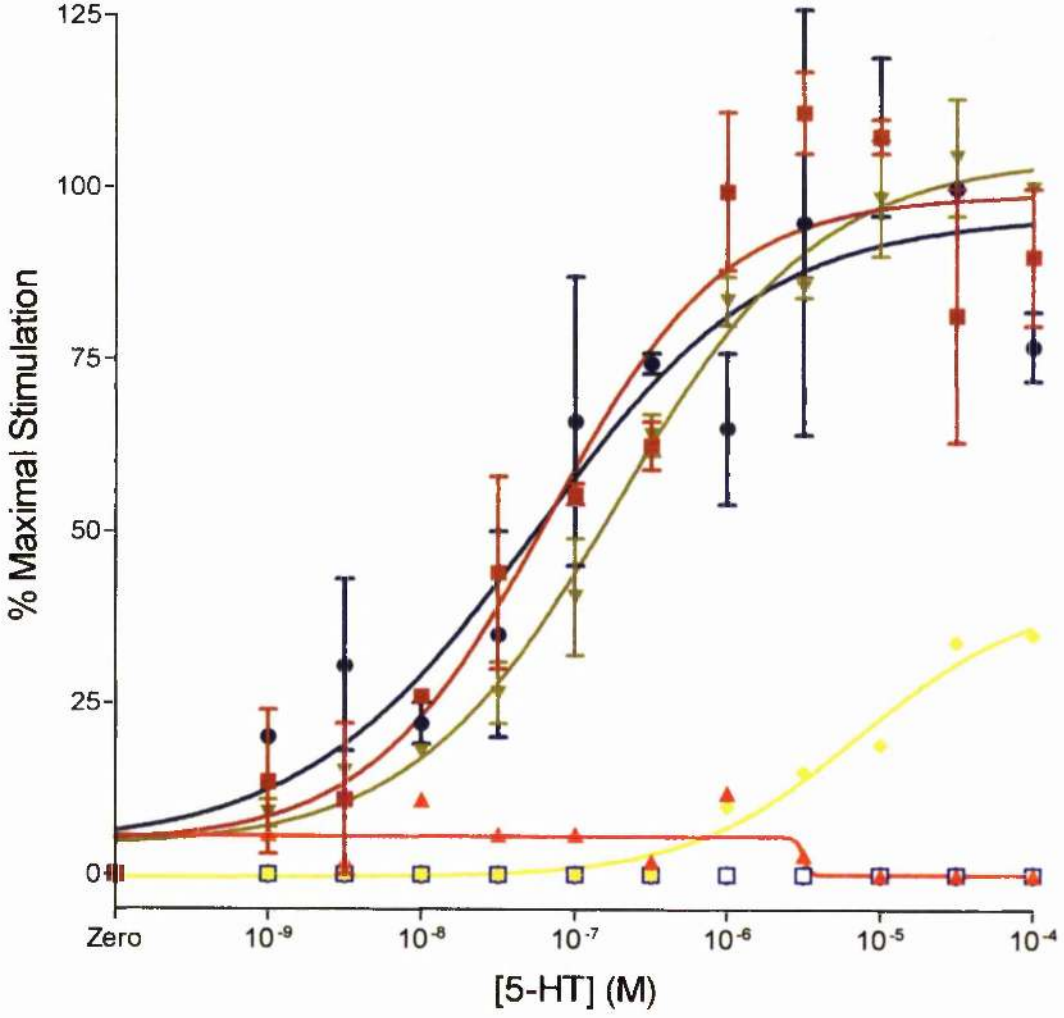

125
100
75
50
25
0
% Maximal Stimulation
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[5-HT] (M)

Figure 5.5 **5-HT concentration response curves for activation of GTPase activity in the 5-HT_{1A} receptor and its mutant $G_{i1\alpha}$ and $G_{o1\alpha}$ pertussis toxin resistant fusion proteins**

Panel A: The potency of 5-HT to stimulate high affinity GTPase activity in both untreated and pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ and 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion proteins was examined.

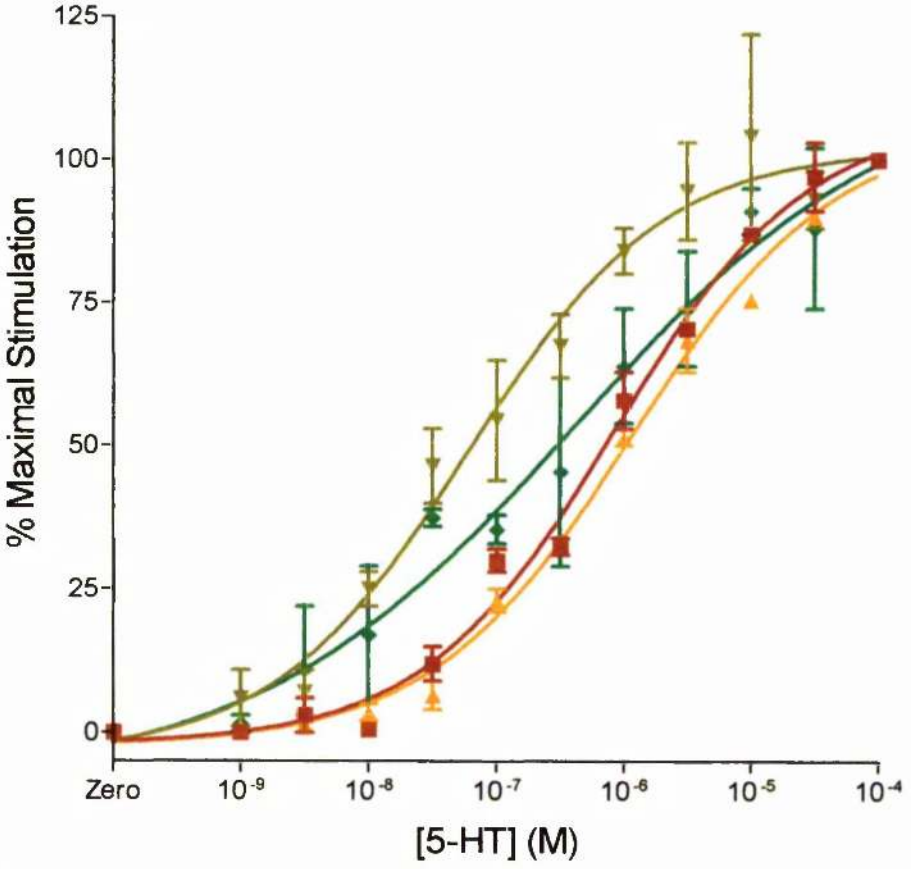
- 5-$HT_{1A}/G_{i1\alpha}C^{351}G$
- 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox
- 5-$HT_{1A}/G_{i1\alpha}C^{351}I$
- 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox

Panel B: 5-HT stimulation of high affinity GTPase activity in both untreated and pertussis toxin-treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins were examined.

- 5-$HT_{1A}/G_{o1\alpha}C^{351}G$
- 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ ptox
- 5-$HT_{1A}/G_{o1\alpha}C^{351}I$
- 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ ptox

Data are mean+/-S.D. of triplicate determinations and are representative of at least three similar experiments.

A


125
100
75
50
25
0
% Maximal Stimulation
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[5-HT] (M)

B

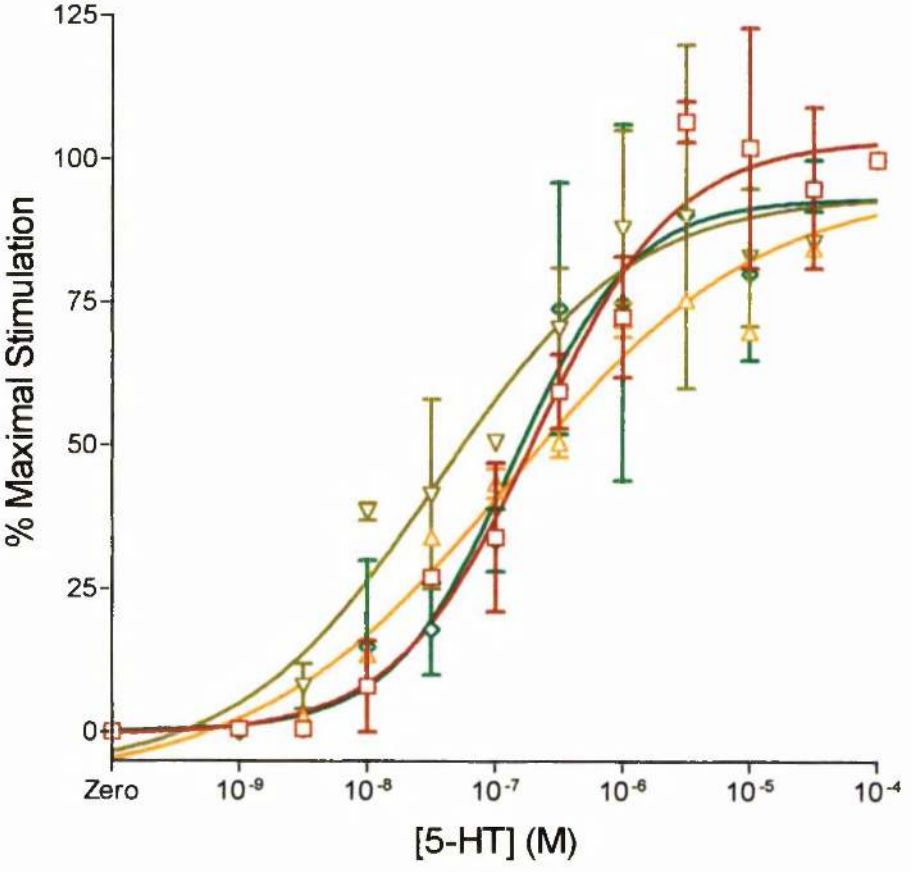
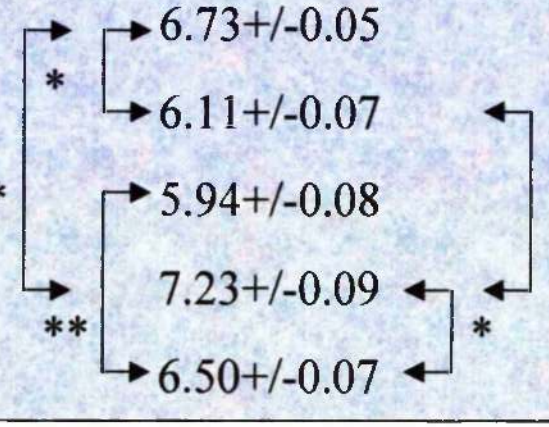

125
100
75
50
25
0
% Maximal Stimulation
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[5-HT] (M)

Table 5.1 **Mean pEC_{50} values from 5-HT stimulation of high affinity GTPase activity for the 5-HT_{1A} receptor-$G_{i1\alpha}$ WT and mutant fusion proteins**

The effects of increasing concentrations of 5-HT on high affinity GTPase activity in membranes expressing the WT and mutant $G_{i1\alpha}$-5-HT_{1A} receptor fusion proteins were measured in terms of pEC_{50}. Brackets indicate statistically significant comparisons. Data are mean +/- SEM from three similar experiments.

Construct Expressed	pEC_{50} (nM) Mean +/-SEM	Number of Replicates
5-HT_{1A}/$G_{i1\alpha}$	6.73+/-0.05	3
5-HT_{1A}/$G_{i1\alpha}C^{351}G$	6.11+/-0.07	3
5-HT_{1A}/$G_{i1\alpha}C^{351}G$ ptox	5.94+/-0.08	3
5-HT_{1A}/$G_{i1\alpha}C^{351}I$	7.23+/-0.09	3
5-HT_{1A}/$G_{i1\alpha}C^{351}I$ ptox	6.50+/-0.07	3



*	$P < 0.05$
**	$P < 0.01$
***	$P < 0.001$

Table 5.2 **Mean pEC_{50} values from 5-HT stimulation of high affinity GTPase activity for the 5-HT_{1A} receptor-$G_{o1\alpha}$ WT and mutant fusion proteins**

The effects of increasing concentrations of 5-HT on high affinity GTPase activity in membranes expressing the WT and mutant $G_{o1\alpha}$-5-HT_{1A} receptor fusion proteins were measured in terms of pEC_{50}. Brackets indicate statistically significant comparisons. Data are mean +/- SEM from three similar experiments.

Construct Expressed		pEC_{50} (nM) Mean +/-SEM		Number of Replicates
5-$HT_{1A}/G_{o1\alpha}$		7.51+/-0.22	***	3
5-$HT_{1A}/G_{o1\alpha}C^{351}G$		6.69+/-0.01		3
5-$HT_{1A}/G_{o1\alpha}C^{351}G$ ptox	***	6.88+/-0.07		3
5-$HT_{1A}/G_{o1\alpha}C^{351}I$		7.46+/-0.03		3
5-$HT_{1A}/G_{o1\alpha}C^{351}I$ ptox		6.71+/-0.22		3

*	$P < 0.05$
**	$P < 0.01$
***	$P < 0.001$

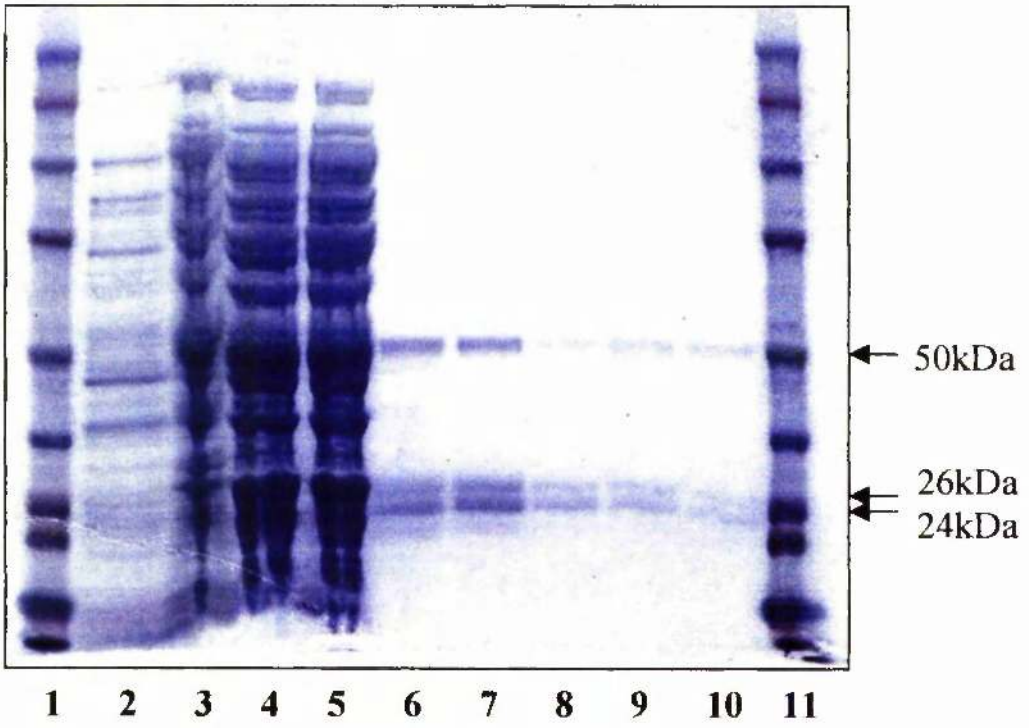
Figure 5.6 **Coommassie Blue staining for purified RGS1- and RGS16-GST following protein purification using Glutathione Sepharose 4B gel**

Protein was stained for with Commassie Blue following electrophoresis of purified protein and crude bacterial extracts using NuPAGE 4-12% Bis-Tris pre-cast gels.

Panel A: Protein of an apparent M_r of approximately 50kDa was apparent, consistent with the calculated size of the RGS1-GST fusion protein. Lanes 1 and 12 contain rainbow markers, lane 2 contains bacterial sample prior to IPTG induction, lanes 3-5 contain bacterial samples at 1, 2 and 3 hours post induction, and lanes 6-10 contain samples of purified RGS16 protein. The protein at approximately 24 and 26kDa corresponds to degraded RGS16 protein and GST.

Panel B: Protein of an apparent M_r of approximately 50kDa was apparent, consistent with the calculated size of the RGS1-GST fusion protein. Lane8 contains rainbow markers, lanes 1, 2, 9 and 10 contain bacterial samples 2 hours (1 + 9) and 3 hours (2 + 10) post IPTG induction, and lanes 3-7 and 11-15 contain samples of purified RGS 16 protein.

A


50kDa
26kDa
24kDa
1 2 3 4 5 6 7 8 9 10 11

B

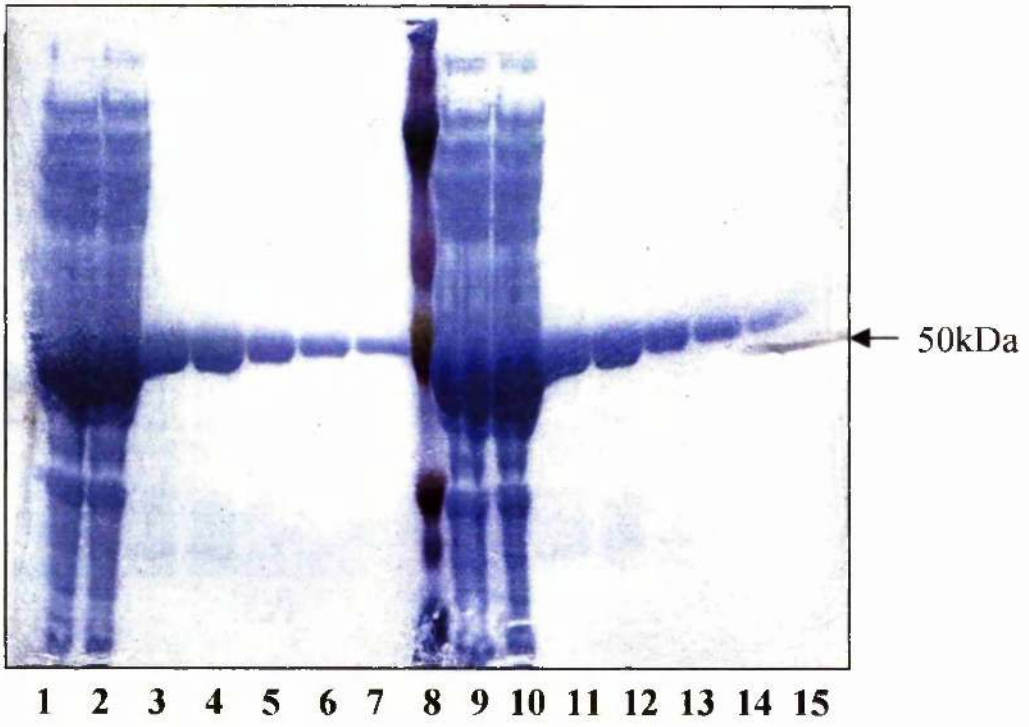

50kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 5.7 **Basal and agonist stimulated high affinity GTPase activity in membranes expressing the 5-HT_{1A} receptor fused to Gly^{351} and Ile^{351} $G_{i1\alpha}$ protein mutants in the presence of RGS1 and RGS16 proteins**

Basal and agonist stimulated high affinity GTPase activity in pertussis toxin pre-treated membranes expressing either the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ (Panel A) or the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ (Panel B) fusion protein. Stimulation with either 5-HT (100μM) or the 5-HT_{1A} selective agonist 8-OH-DAPT (100μM) was significantly ($P < 0.05$) enhanced by the presence of purified RGS1 or RGS16 proteins (1μM). Data are means of triplicate determinations and are representative of at least three similar experiments.

- 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ ptox
- 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ ptox

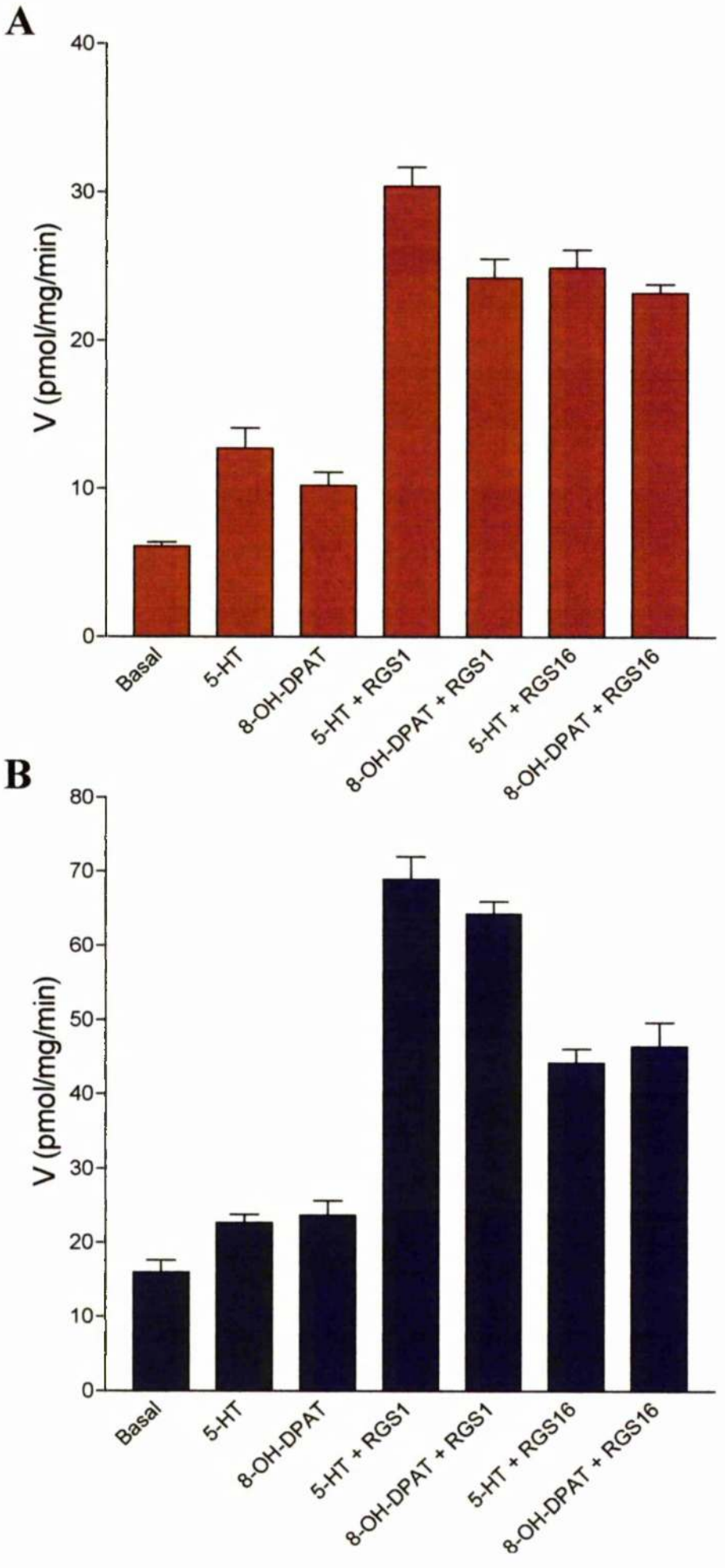
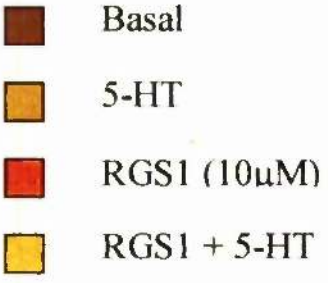

A
V (pmol/mg/min)
0
10
20
30
40
Basal
5-HT
8-OH-DPAT
5-HT + RGS1
8-OH-DPAT + RGS1
5-HT + RGS16
8-OH-DPAT + RGS16
B
V (pmol/mg/min)
0
10
20
30
40
50
60
70
80
Basal
5-HT
8-OH-DPAT
5-HT + RGS1
8-OH-DPAT + RGS1
5-HT + RGS16
8-OH-DPAT + RGS16

Figure 5.8 **Lack of effect of 5-HT and RGS1 protein on mock transfected HEK293 cells**

High affinity GTPase activity was measured in un-transfected HEK293 membranes in the presence and absence of 5-HT (100μM) and RGS1 (10μM). Data are means of triplicate determinations and are representative of at least three similar experiments.



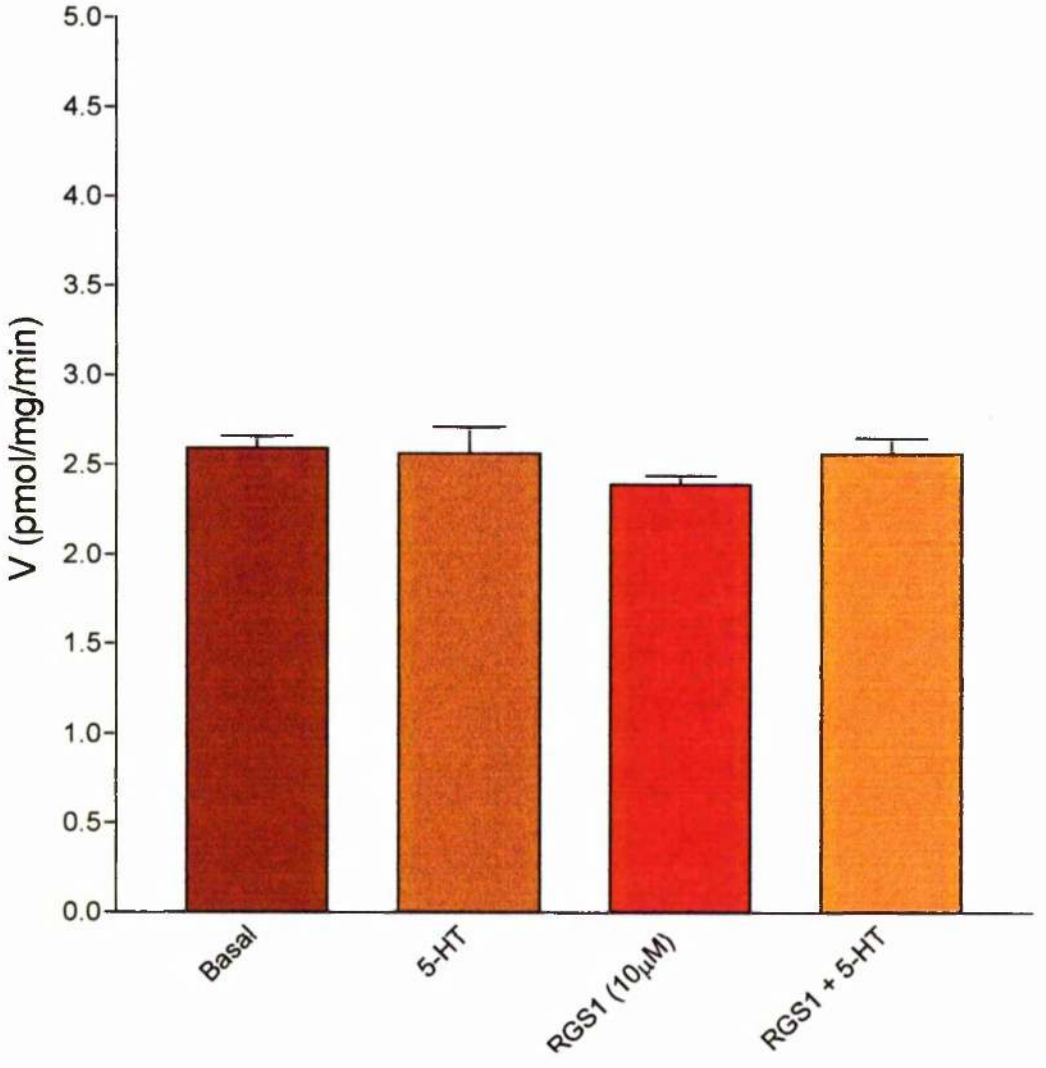
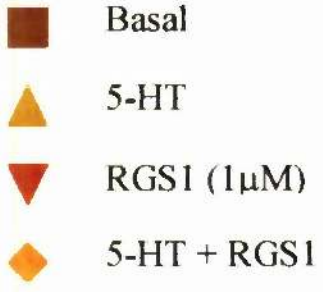

V (pmol/mg/min)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Basal
5-HT
RGS1 (10μM)
RGS1 + 5-HT

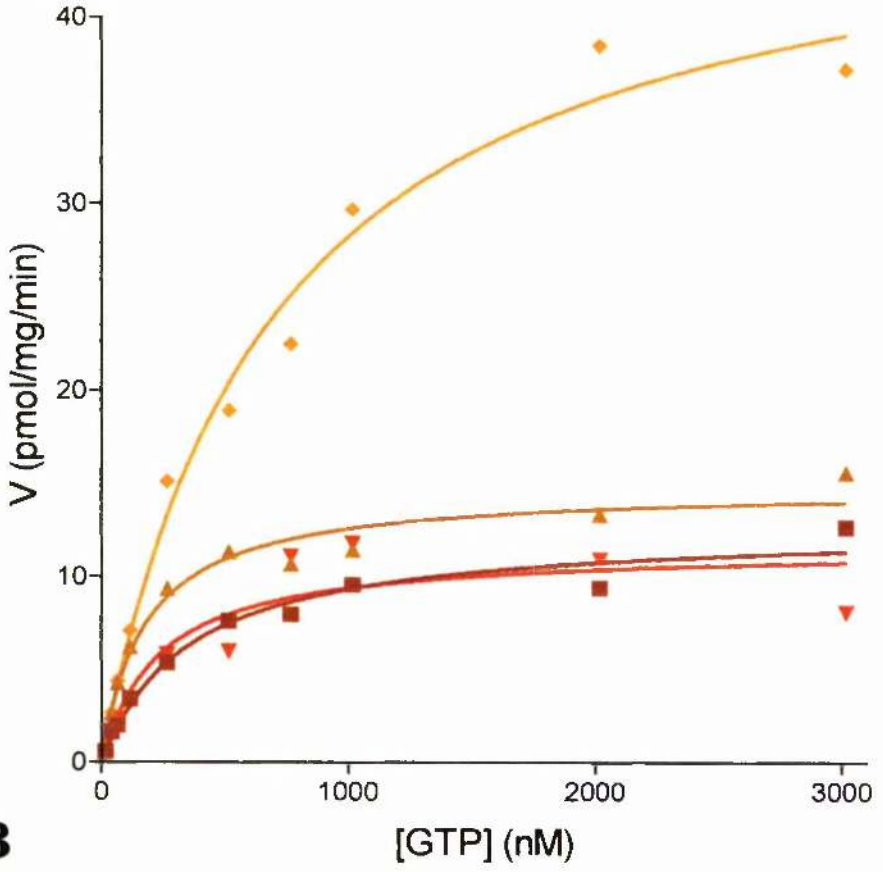
Figure 5.9 **Basal and 5-HT stimulated GTPase activity of membranes expressing the 5-HT_{1A} receptor in the presence and absence of RGS1**

Panel A: GTPase activity of membranes expressing the 5-HT_{1A} receptor with increasing concentrations of GTP as substrate. 5-HT (100μM) stimulated GTPase activity was measured in the presence and absence of RGS1 (1μM). Data were modelled to an equation of a one-site fit hyperbola. Panel B: The data generated were transformed to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.



A


40
30
20
10
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

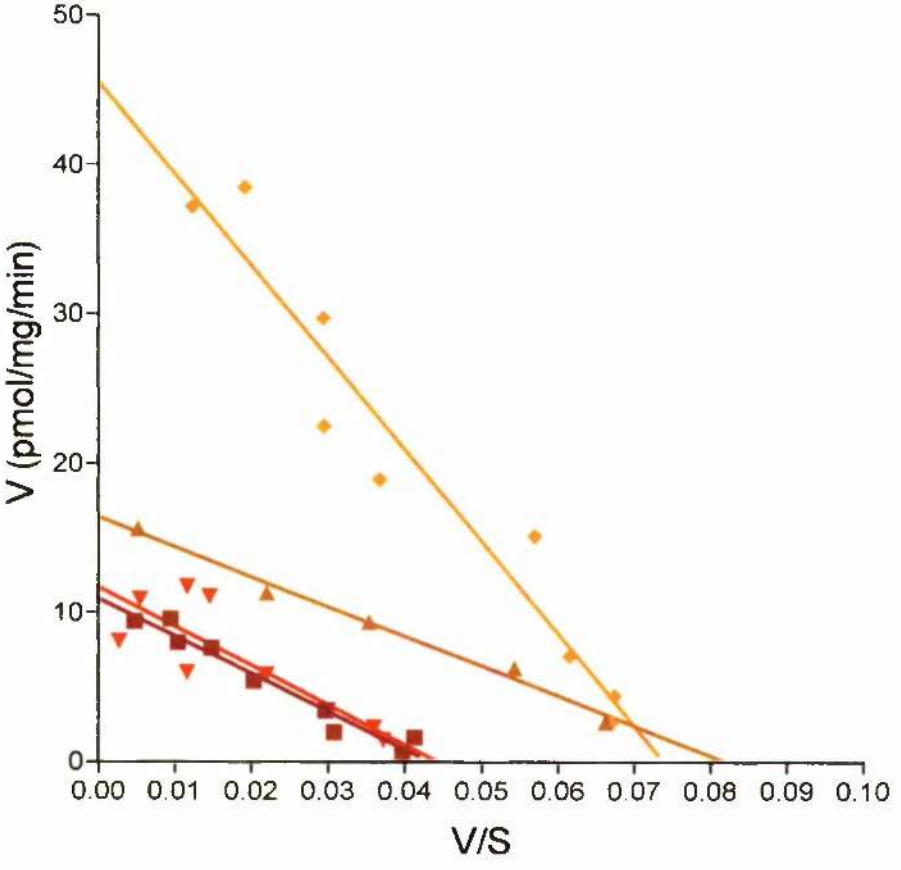
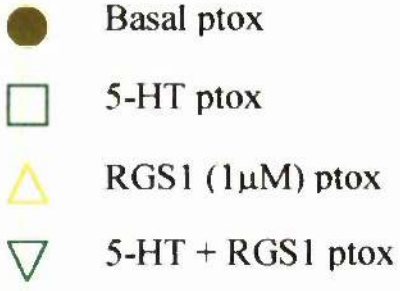

50
40
30
20
10
0
V (pmol/mg/min)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
V/S

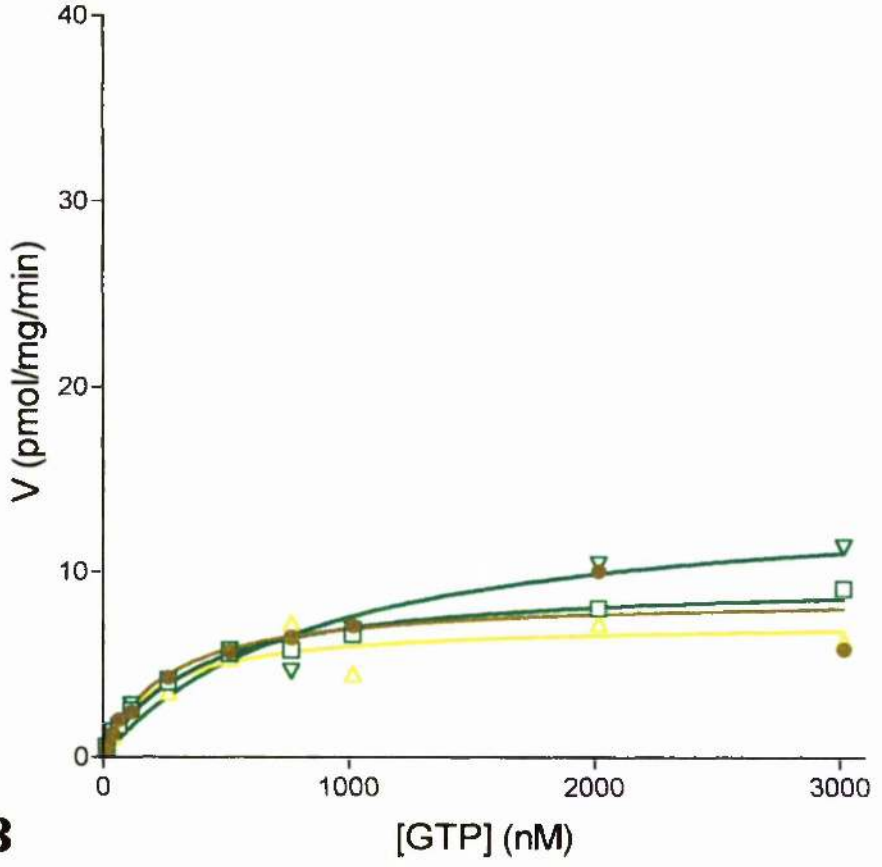
Figure 5.10 **Basal and 5-HT stimulated GTPase activity of pertussis toxin-treated membranes expressing the 5-HT_{1A} receptor in the presence and absence of RGS1**

Panel A: GTPase activity in pertussis toxin-treated membranes expressing the 5-HT_{1A} receptor with increasing concentrations of GTP as substrate. 5-HT (100μM) stimulated GTPase activity was measured in the presence and absence of RGS1 (1μM). Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.



A


40
30
20
10
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

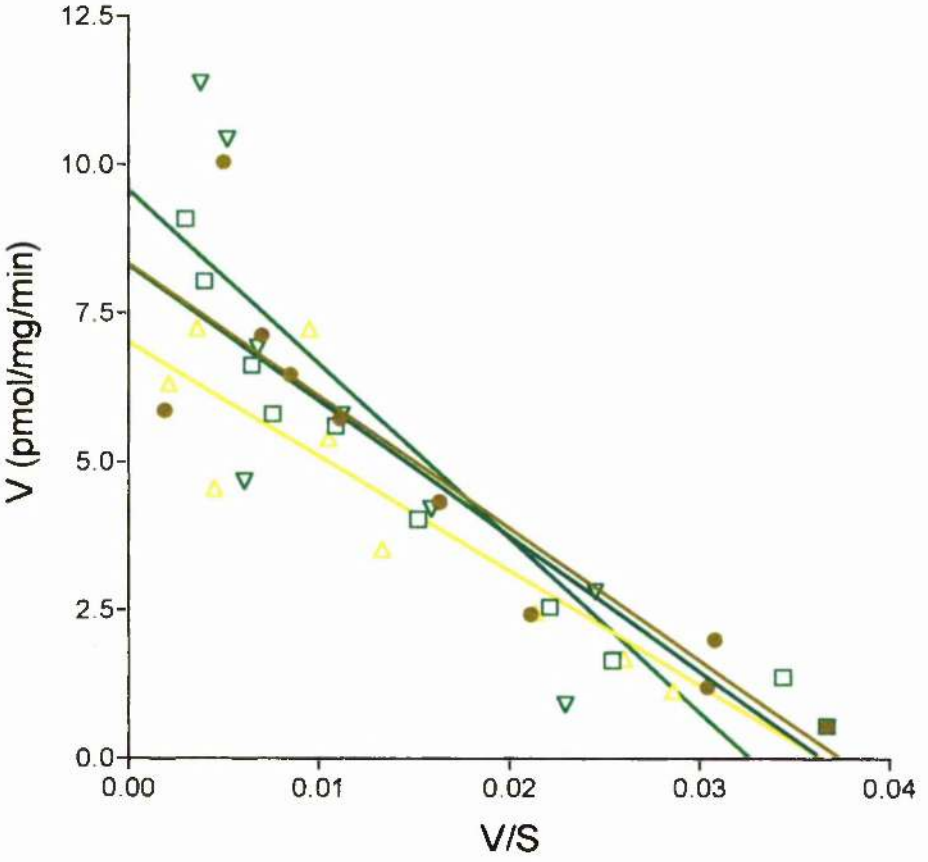

12.5
10.0
7.5
5.0
2.5
0.0
V (pmol/mg/min)
0.00
0.01
0.02
0.03
0.04
V/S

Figure 5.11 **Basal and 5-HT stimulated GTPase activity of membranes expressing the 5-$HT_{1A}G_{i1\alpha}$ and 5-$HT_{1A}/G_{o1\alpha}$ receptors in the presence and absence of RGS1**

Panel A: GTPase activity in membranes expressing the 5-$HT_{1A}G_{i1\alpha}$ fusion protein with increasing concentrations of GTP as substrate. 5-HT (100μM) stimulated GTPase activity was measured in the presence and absence of RGS1 (1μM). Panel B: GTPase activity in membranes expressing the 5-$HT_{1A}G_{o1\alpha}$ fusion protein with increasing concentrations of GTP as substrate. 5-HT (100μM) stimulated GTPase activity was measured in the presence and absence of RGS1 (1μM).

Data are means of triplicate determinations and are representative of at least three similar experiments.

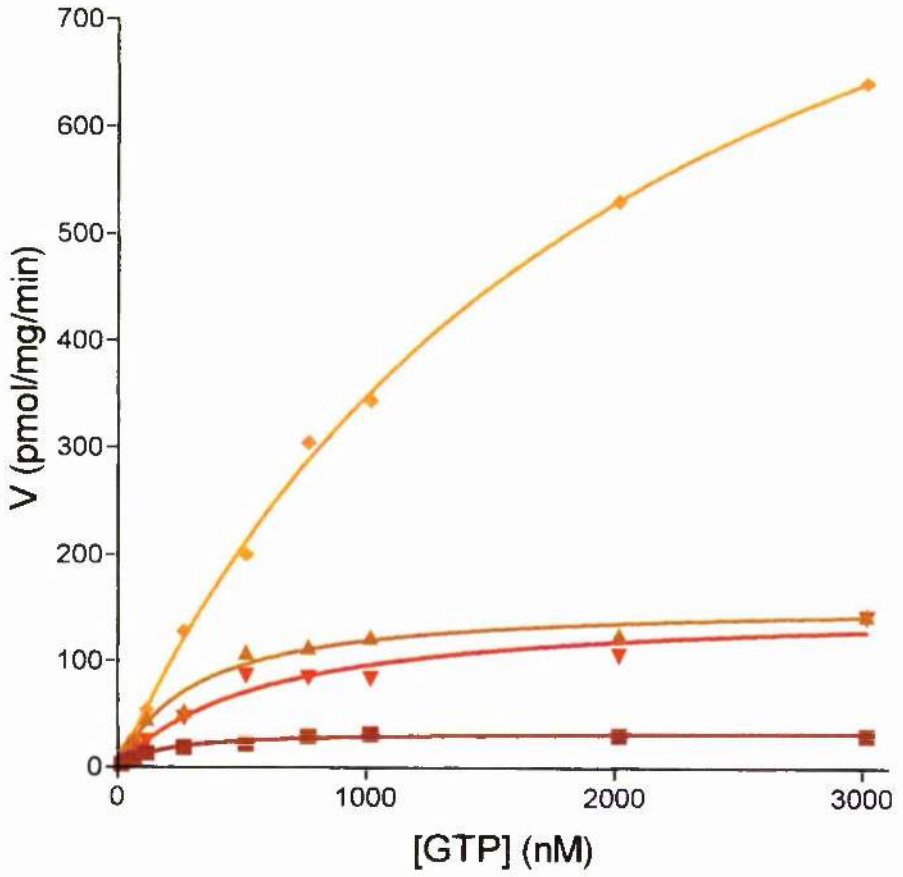
Basal

5-HT

RGS1 (1μM)

5-HT + RGS1

A


700
600
500
400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

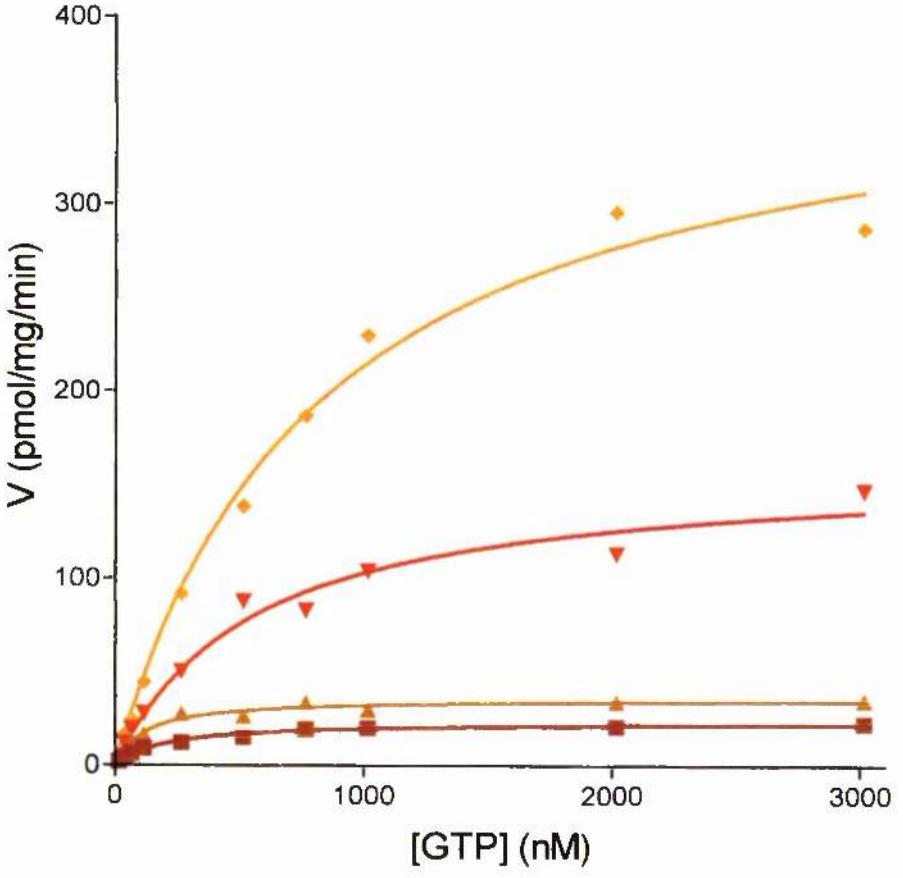
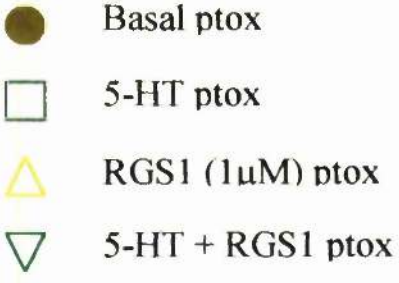

400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

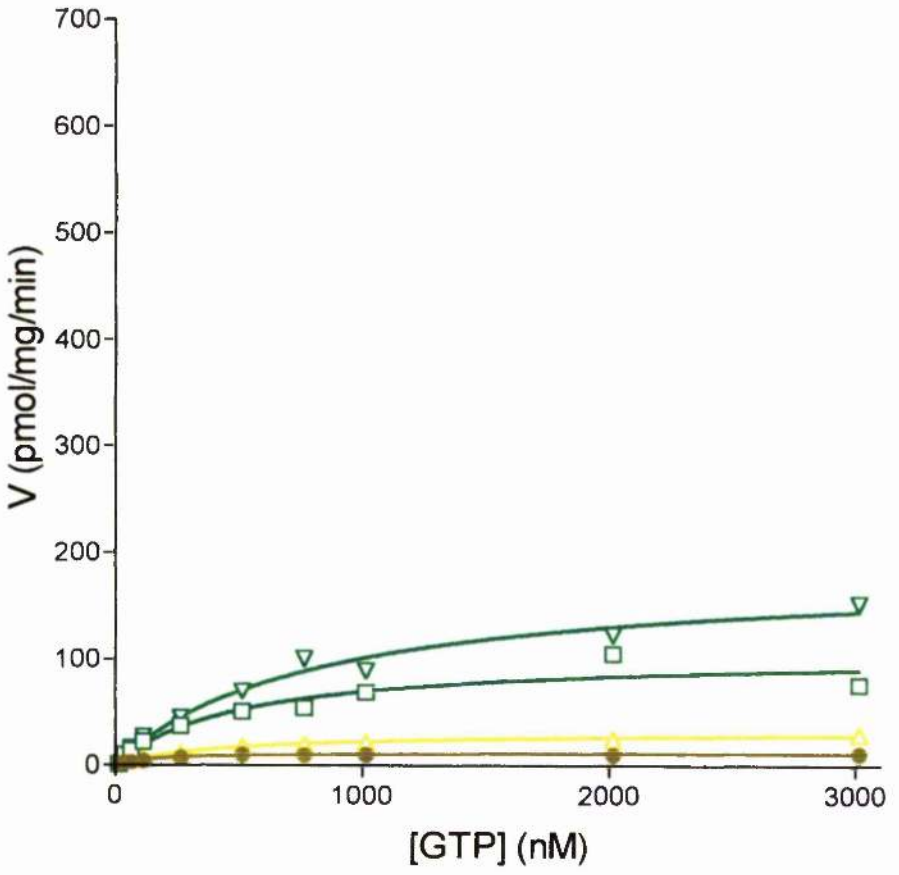
Figure 5.12 **Basal and 5-HT stimulated GTPase activity of pertussis toxin-treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}$ and 5-$HT_{1A}/G_{o1\alpha}$ receptors in the presence and absence of RGS1**

Panel A: GTPase activity in pertussis toxin-treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}$ fusion protein with increasing concentrations of GTP as substrate. 5-HT (100μM) stimulated GTPase activity was measured in the presence and absence of RGS1 (1μM). Panel B: GTPase activity in pertussis toxin-treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}$ fusion protein with increasing concentrations of GTP as substrate. 5-HT (100μM) stimulated GTPase activity was measured in the presence and absence of RGS1 (1μM).

Data are means of triplicate determinations and are representative of at least three similar experiments.



A


700
600
500
400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

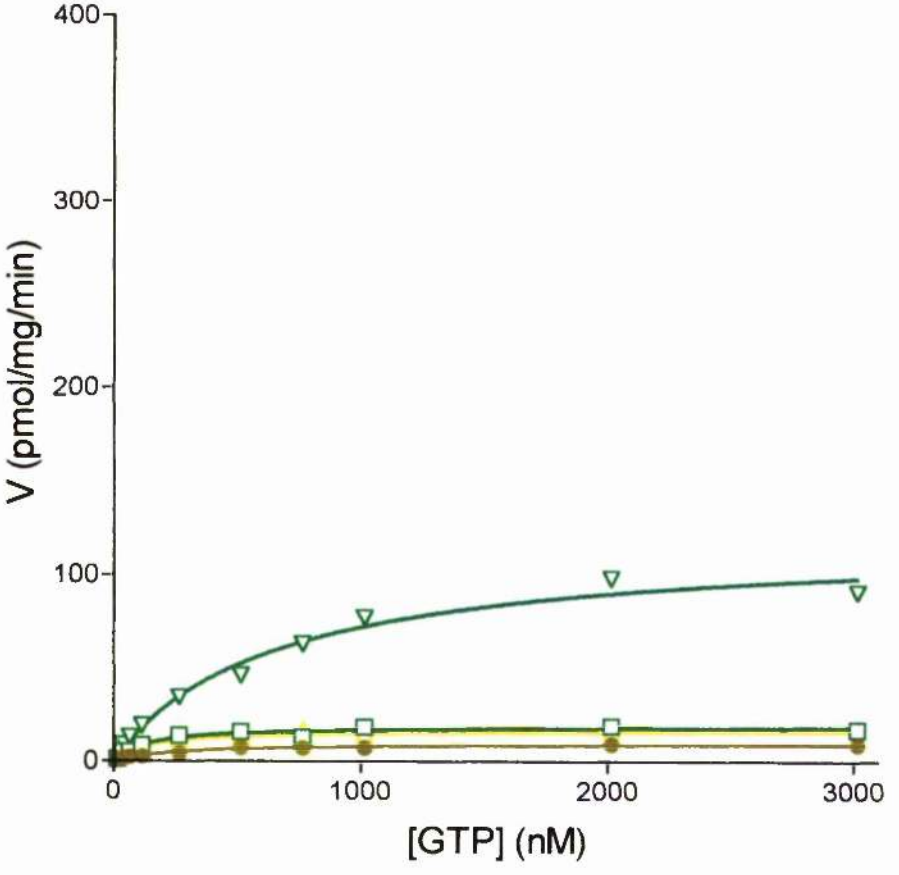
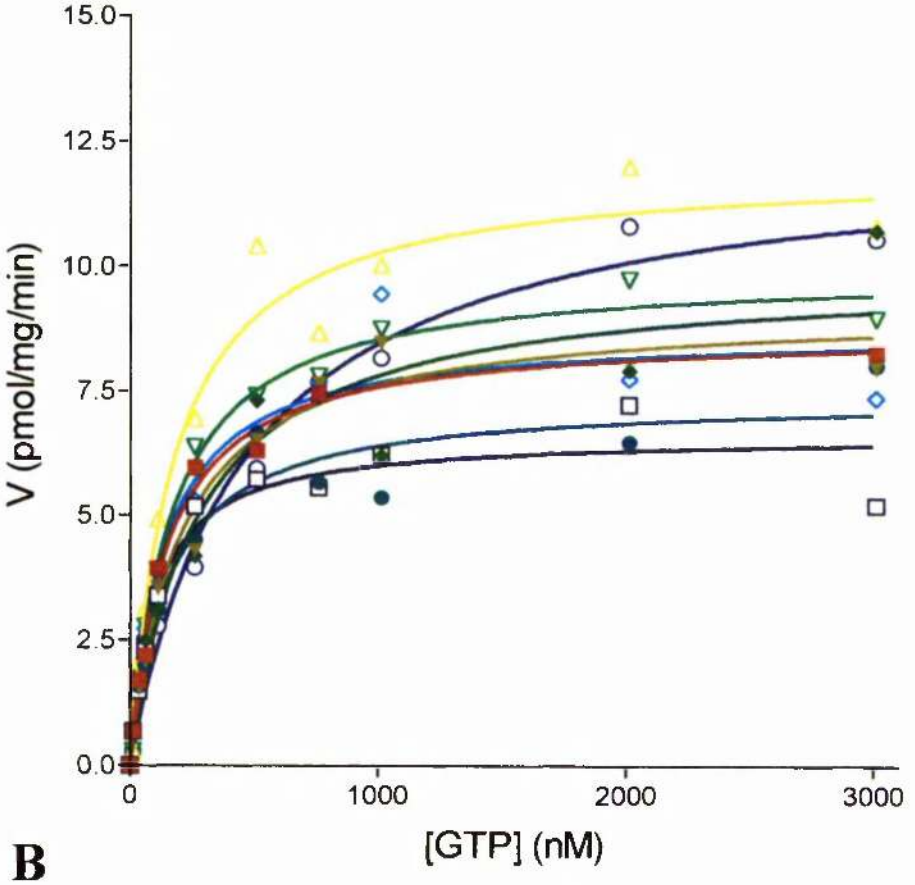

400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

Figure 5.13 **Effects of increasing concentrations of RGS1 on the high affinity GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G$ fusion protein with increasing concentrations of GTP as substrate. Basal GTPase activity was measured in the presence of various concentrations of RGS1. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


15.0
12.5
10.0
7.5
5.0
2.5
0.0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

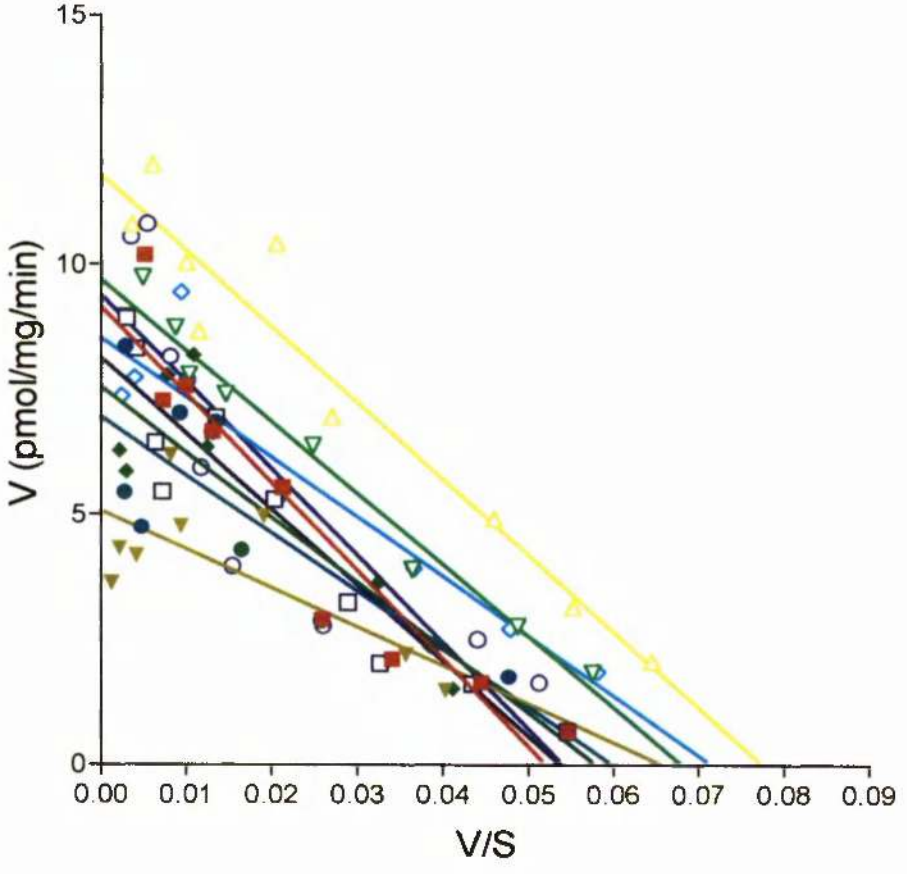

15
10
5
0
V (pmol/mg/min)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
V/S

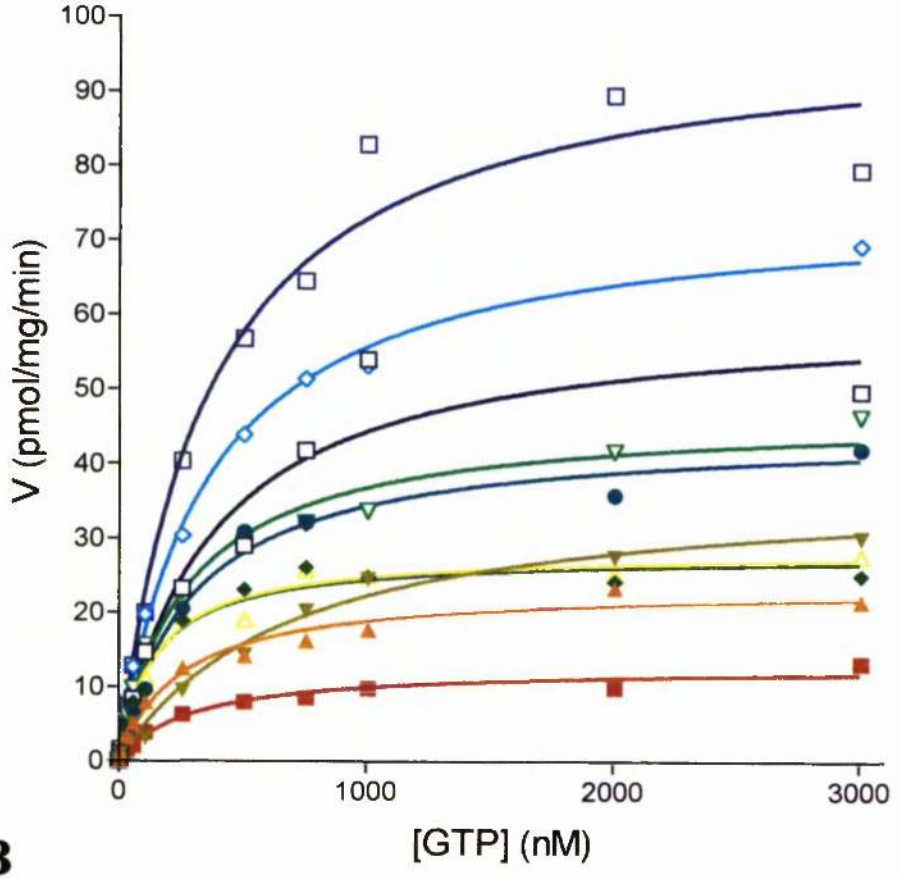
Figure 5.14 **Effects of increasing concentrations of RGS1 on the 5-HT stimulated GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G$ fusion protein with increasing concentrations of GTP as substrate. 5-HT stimulated GTPase activity was measured in the presence of various concentrations of RGS1. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

- Basal
- 5-HT
- 5-HT + RGS1 1nM
- 5-HT + RGS1 5nM
- 5-HT + RGS1 10nM
- 5-HT + RGS1 50nM
- 5-HT + RGS1 100nM
- 5-HT + RGS1 500nM
- 5-HT + RGS1 1μM
- 5-HT + RGS1 5μM

A


100
90
80
70
60
50
40
30
20
10
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

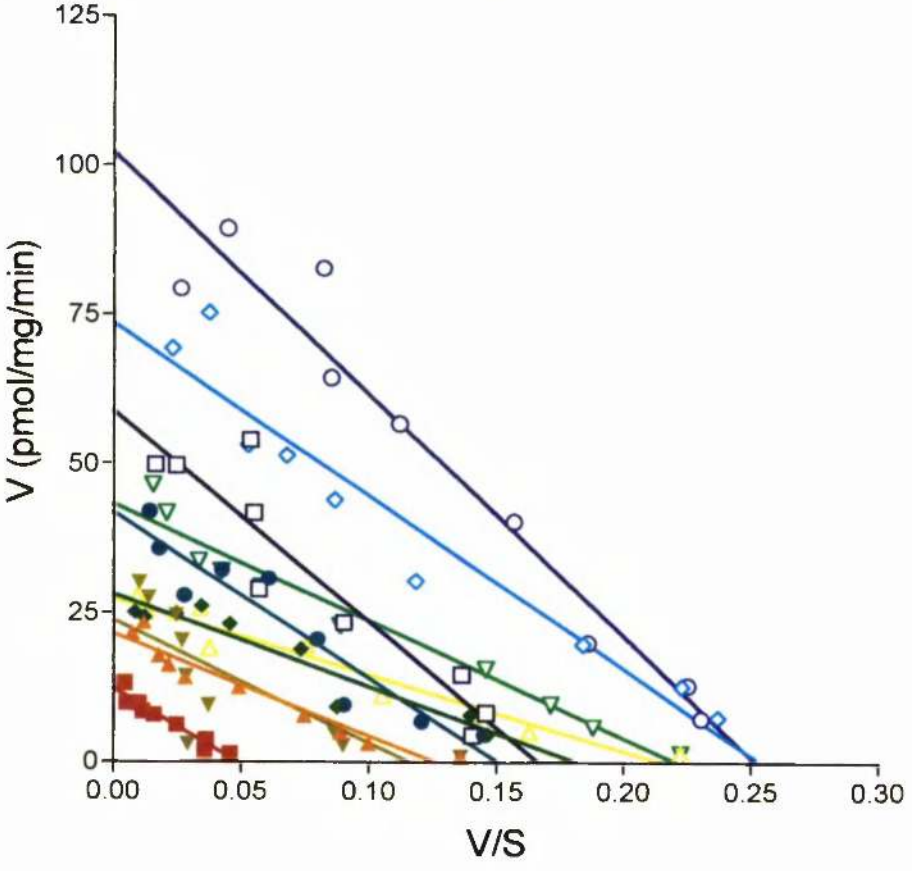

125
100
75
50
25
0
V (pmol/mg/min)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
V/S

Figure 5.15 **Effects of increasing concentrations of RGS16 on the high affinity GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G$ fusion protein with increasing concentrations of GTP as substrate. Basal GTPase activity was measured in the presence of various concentrations of RGS16. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

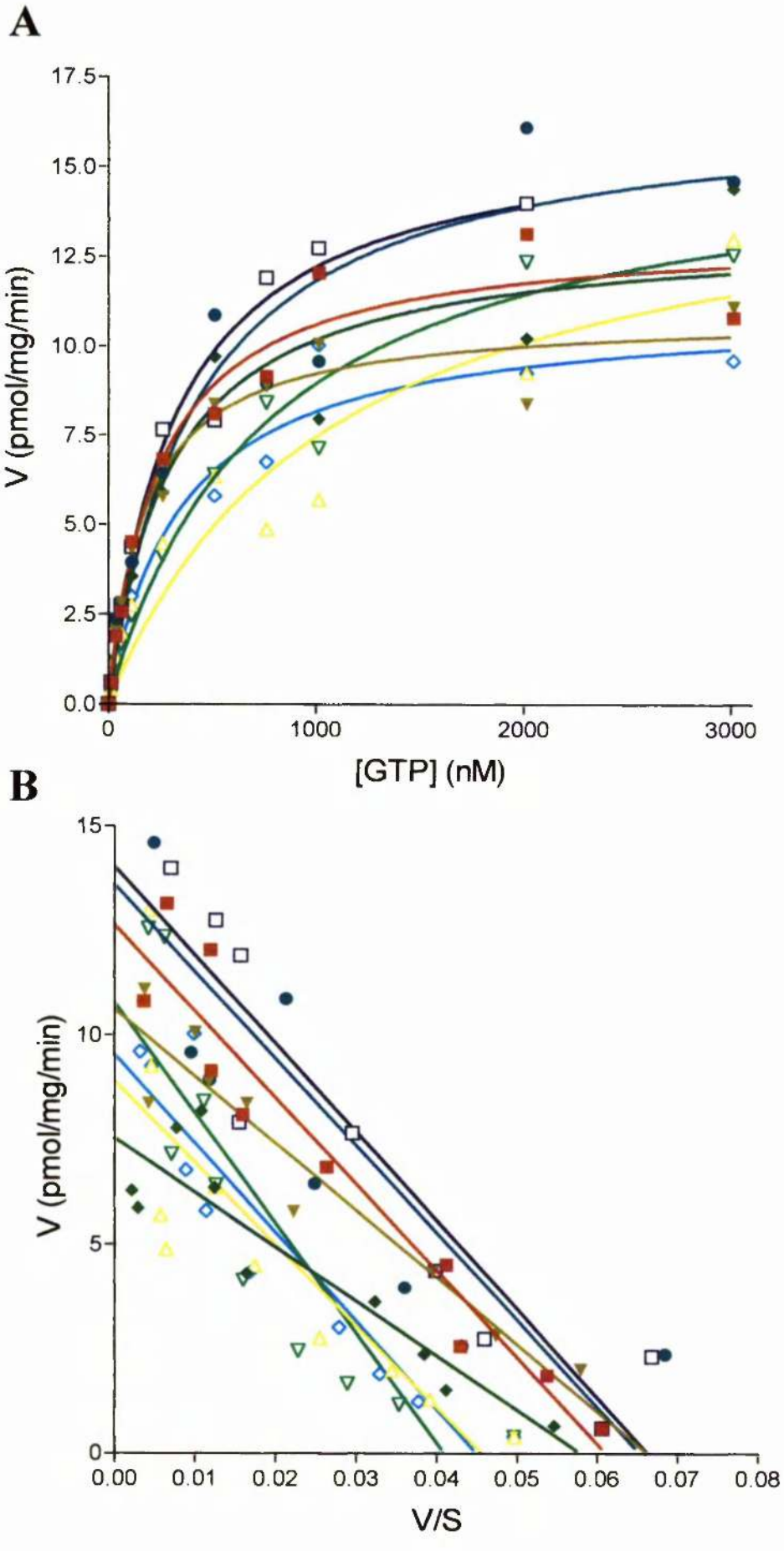
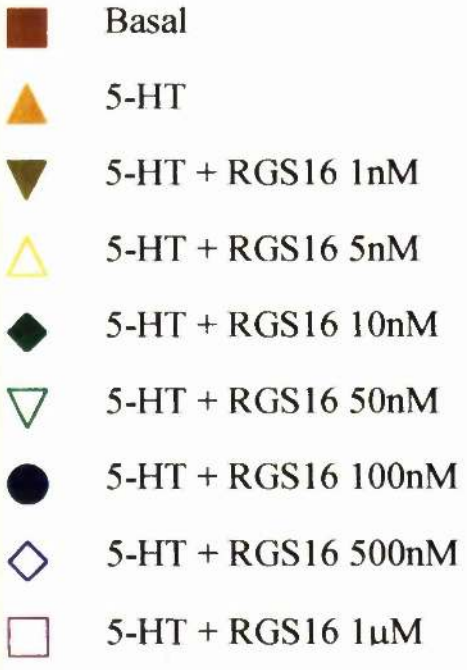

A
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)
B
15
10
5
0
V (pmol/mg/min)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
V/S

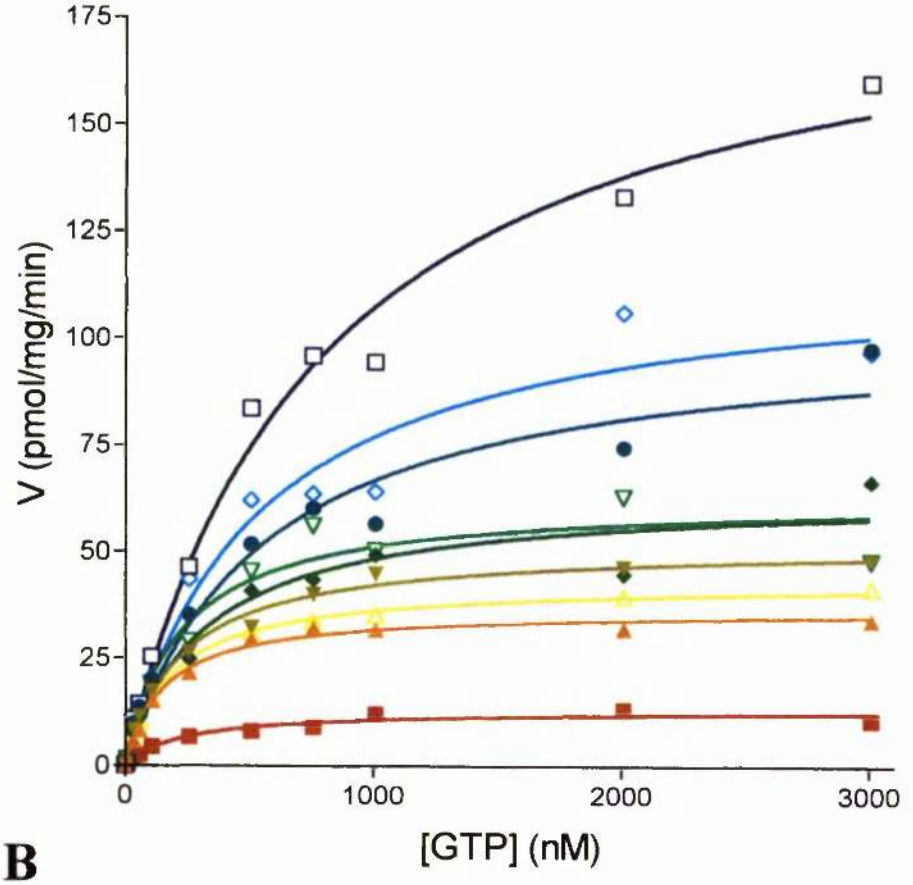
Figure 5.16 **Effects of increasing concentrations of RGS16 on the 5-HT stimulated GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G$ fusion protein with increasing concentrations of GTP as substrate. 5-HT stimulated GTPase activity was measured in the presence of various concentrations of RGS16. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.



A


175
150
125
100
75
50
25
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

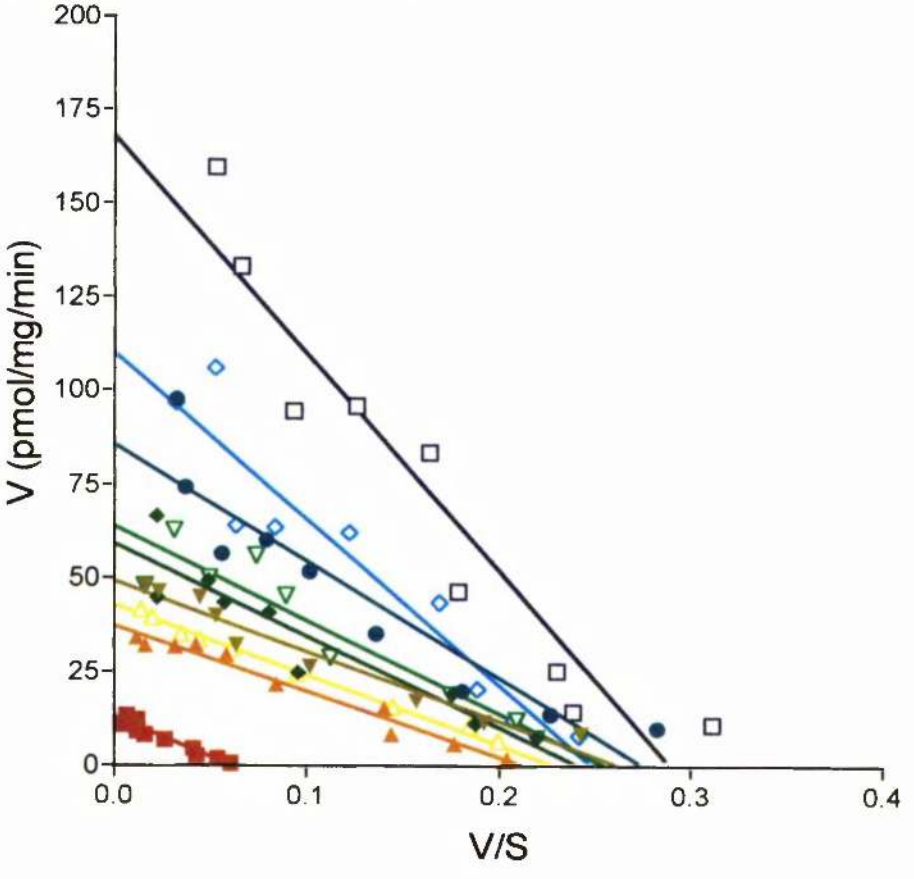

200
175
150
125
100
75
50
25
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
V/S

Table 5.3 **Mean K_m and V_{max} values for RGS protein concentration response high affinity GTPase assays with the 5-HT_{1A}/$G_{i1\alpha}C^{351}G$ fusion protein**

Panel A: Effects of a range of RGS1 and RGS16 protein concentrations on the K_m and V_{max} of basal high affinity GTPase activity in membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}G$ fusion protein.

Panel B: Effects of a range of RGS1 and RGS16 protein concentrations on the K_m and V_{max} of 5-HT stimulated high affinity GTPase activity in membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}G$ fusion protein.

Data are mean +/- SEM from three similar experiments. An * denotes a result significantly different from the 0nM RGS protein concentration ($P < 0.05$). Values are in nM.

A

	RGS1		RGS16	
[RGS]	**Km**	**Vmax**	**Km**	**Vmax**
0nM (basal)	309+/-88	11.3+/-0.8	269+/-61	11.2+/-1.0
1nM	141+/-86	6.6+/-0.8	257+/-50	13.0+/-2.0
5nM	160+/-11	11.4+/-0.6	588+/-239	11.7+/-2.2
10nM	420+/-228	10.0+/-1.5	494+/-239	16.0+/-2.1
50nM	208+/-53	11.3+/-1.0	358+/-200	10.0+/-3.0
100nM	232+/-85	7.6+/-0.3	467+/-54	19.0+/-2.5
500nM	152+/-14	9.4+/-0.6	595+/-143	14.7+/-2.0
1μM	179+/-39	7.5+/-0.6	706+/-453	22.3+/-7.9
5μM	466+/-28	15.1+/-1.4		

B

	RGS1		RGS16	
[RGS]	**Km**	**Vmax**	**Km**	**Vmax**
0nM (5-HT)	201+/-25	29.8+/-2.4	235+/-43	40.5+/-4.9
1nM+5-HT	465+/-172	29.3+/-5.2	223+/-8	43.0+/-4.5
5nM+5-HT	175+/-14	34.3+/-3.2	198+/-24	42.3+/-1.2
10nM+5-HT	216+/-38	25.0+/-1.5	321+/-66	54.3+/-9.2
50nM+5-HT	256+/-25	49.7+/-2.7*	274+/-38	64.0+/-1.0*
100nM+5-HT	346+/-27	38.0+/-6.0*	404+/-78*	68.0+/-18.1*
500nM+5-HT	428+/-62	89.3+/-6.8*	381+/-83*	101.0+/-10.4*
1μM +5-HT	451+/-197*	48.0+/-6.7*	563+/-125*	116.7+/-38.2*
5μM +5-HT	443+/-59*	110.7+/-7.6*		

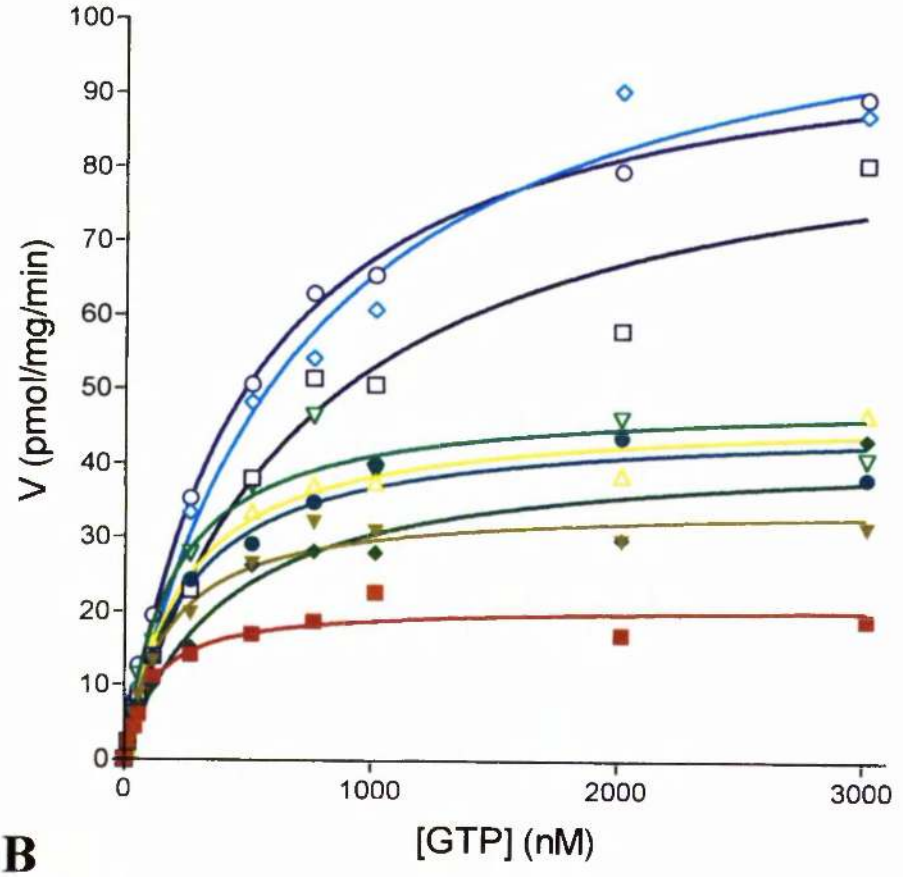
$P < 0.05$

Figure 5.17 **Effects of increasing concentrations of RGS1 on the high affinity GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. Basal GTPase activity was measured in the presence of various concentrations of RGS1. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


100
90
80
70
60
50
40
30
20
10
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

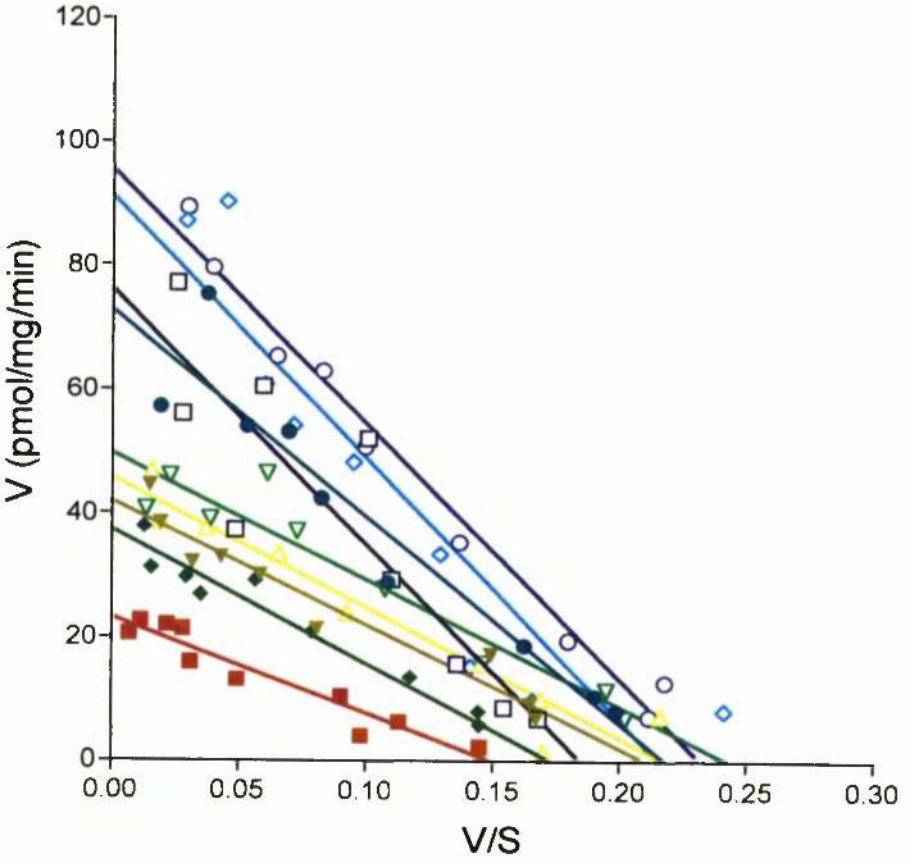

120
100
80
60
40
20
0
V (pmol/mg/min)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
V/S

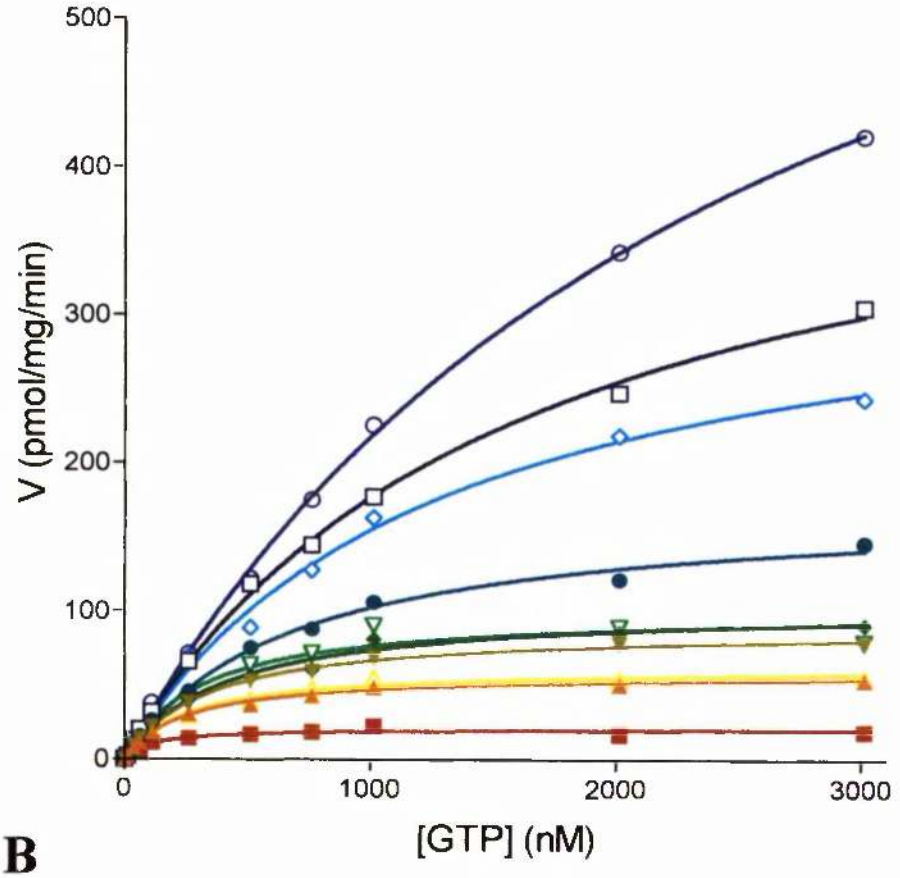
Figure 5.18 **Effects of increasing concentrations of RGS1 on the 5-HT stimulated GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. 5-HT stimulated GTPase activity was measured in the presence of various concentrations of RGS1. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

- Basal
- 5-HT
- 5-HT + RGS1 1nM
- 5-HT + RGS1 5nM
- 5-HT + RGS1 10nM
- 5-HT + RGS1 50nM
- 5-HT + RGS1 100nM
- 5-HT + RGS1 500nM
- 5-HT + RGS1 1μM
- 5-HT + RGS1 5μM

A


500
400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

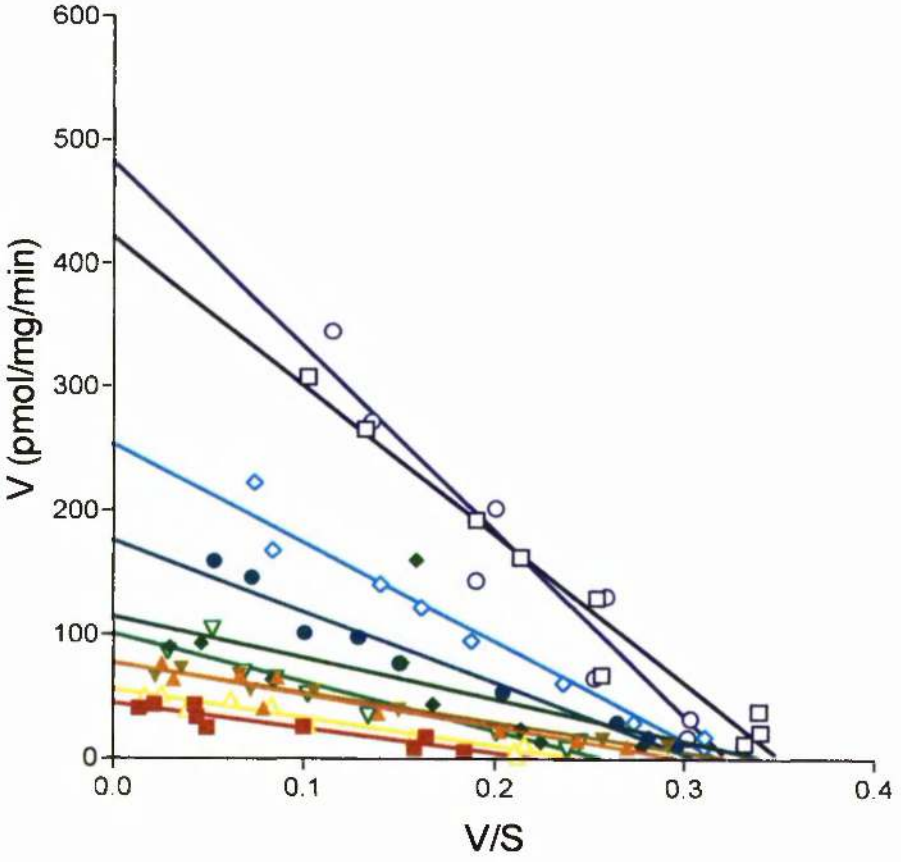
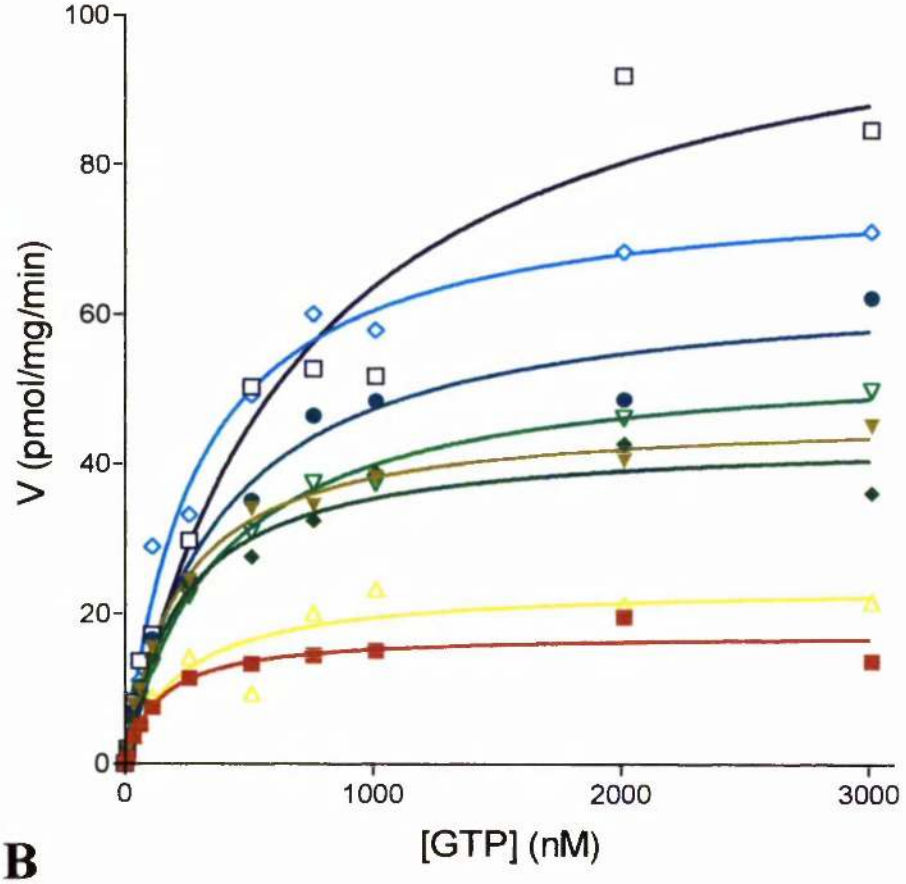

600
500
400
300
200
100
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
V/S

Figure 5.19 **Effects of increasing concentrations of RGS16 on the high affinity GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. Basal GTPase activity was measured in the presence of various concentrations of RGS16. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


100
80
60
40
20
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

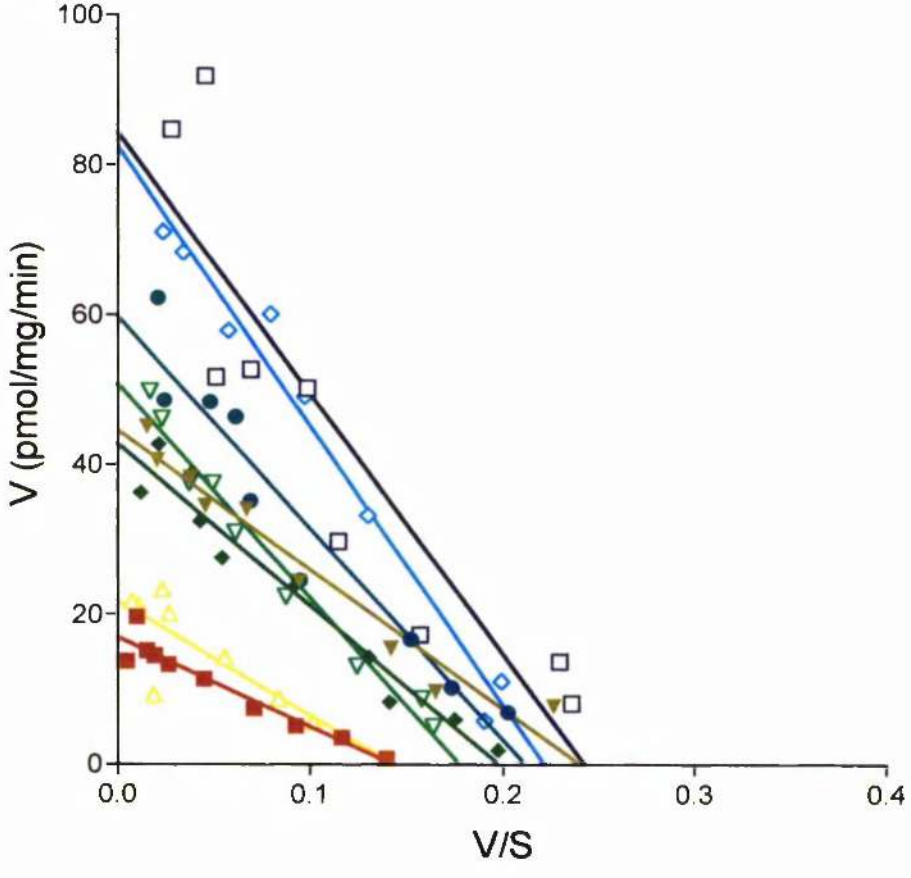

100
80
60
40
20
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
V/S

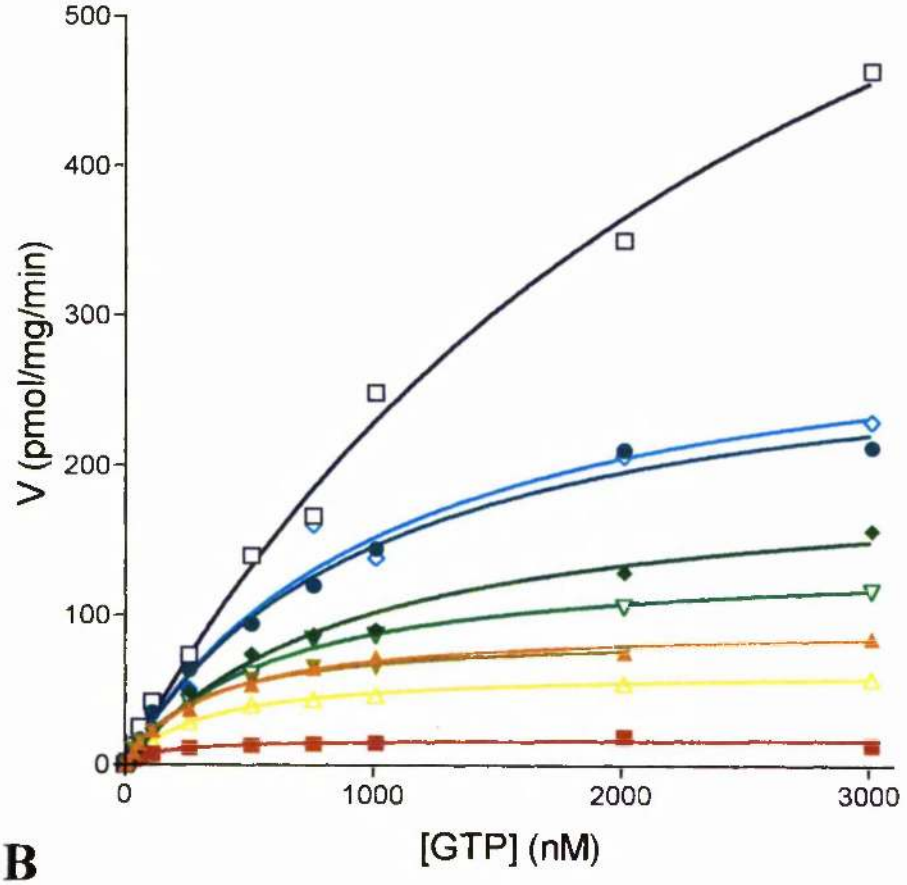
Figure 5.20 **Effects of increasing concentrations of RGS16 on the 5-HT stimulated GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. 5-HT stimulated GTPase activity was measured in the presence of various concentrations of RGS16. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

- Basal
- 5-HT
- 5-HT + RGS16 1nM
- 5-HT + RGS16 5nM
- 5-HT + RGS16 10nM
- 5-HT + RGS16 50nM
- 5-HT + RGS16 100nM
- 5-HT + RGS16 500nM
- 5-HT + RGS16 1μM

A


500
400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

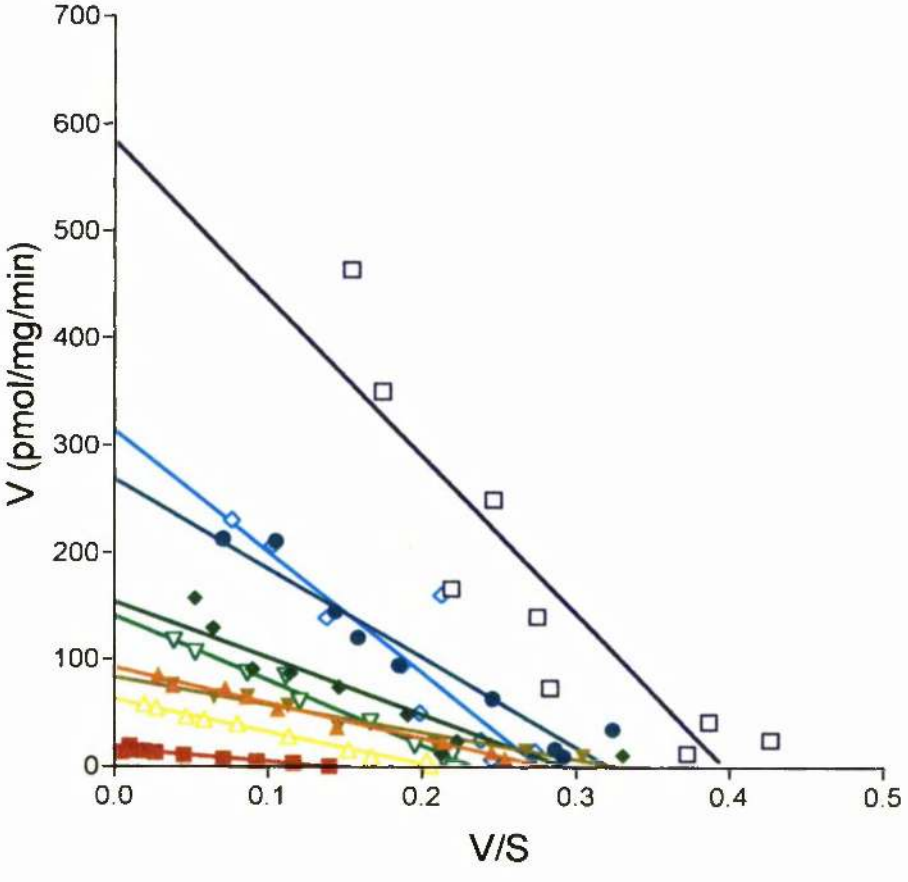

700
600
500
400
300
200
100
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
0.5
V/S

Table 5.4 **Mean K_m and V_{max} values for RGS protein concentration response high affinity GTPase assays with the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein**

Panel A: Effects of a range of RGS1 and RGS16 protein concentrations on the K_m and V_{max} of basal high affinity GTPase activity in membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein.

Panel B: Effects of a range of RGS1 and RGS16 protein concentrations on the K_m and V_{max} of 5-HT stimulated high affinity GTPase activity in membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein.

Data are mean +/- SEM from three similar experiments. An * denotes a result significantly different from the 0nM RGS protein concentration ($P < 0.05$). Values are in nM.

A

	RGS1		RGS16	
[RGS]	**Km**	**Vmax**	**Km**	**Vmax**
0nM (basal)	219+/-36	38.7+/-5.3	210+/-48	38.0+/-9.9
1nM	266+/-62	41.7+/-3.4	320+/-104	58.3+/-8.1
5nM	227+/-20	51.3+/-3.8*	170+/-22	24.7+/-0.7
10nM	325+/-61	42.0+/-2.7*	269+/-77	56.0+/-9.6
50nM	242+/-37	58.3+/-7.9*	317+/-22	53.7+/-2.0
100nM	295+/-36	57.3+/-8.9*	250+/-75	56.7+/-7.4
500nM	520+/-87	96.0+/-10.2*	293+/-27	68.0+/-4.5
1μM	567+/-82	79.0+/-7.2*	411+/-151	81.3+/-14.9
5μM	447+/-69	89.7+/-13.9*		

$P < 0.05$

B

	RGS1		RGS16	
[RGS]	**Km**	**Vmax**	**Km**	**Vmax**
0nM (5-HT)	219+/-35	76.0+/-8.7	277+/-67	56.5+/-11.3
1nM+5-HT	265+/-62	83.7+/-3.5	639+/-144	107.0+/-18.4*
5nM+5-HT	227+/-20	66.7+/-8.8	394+/-42*	67.0+/-2.1*
10nM+5-HT	325+/-61	105.3+/-9.3	587+/-203*	120.3+/-38.4*
50nM+5-HT	242+/-37	130.3+/-24.1	925+/-231*	180.7+/-23.4*
100nM+5-HT	295+/-36*	182.3+/-8.8*	834+/-93*	189.3+/-53.3*
500nM+5-HT	520+/-87*	351.0+/-43.0*	1676+/-396*	389.7+/-74.7*
1μM +5-HT	567+/-87*	429.7+/-18.5*	1682+/-658*	468.3+/-219*
5μM +5-HT	447+/-69*	672.7+/-69.4*		

$P < 0.05$

Figure 5.21 **RGS1 and RGS16 regulation of basal and 5-HT stimulated GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}G/I$ fusion proteins**

Panel A: Utilising the V_{max} for GTP hydrolysis and B_{max} for expression levels, turnover numbers for basal GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}G/I$ fusion proteins in the presence of increasing concentrations of purified RGS1 and RGS16 were plotted. Panel B: Turnover numbers were plotted for 5-HT stimulated GTPase activity in the presence of various concentrations of RGS1 and 16.

Data are means of triplicate determinations and are representative of at least three similar experiments.

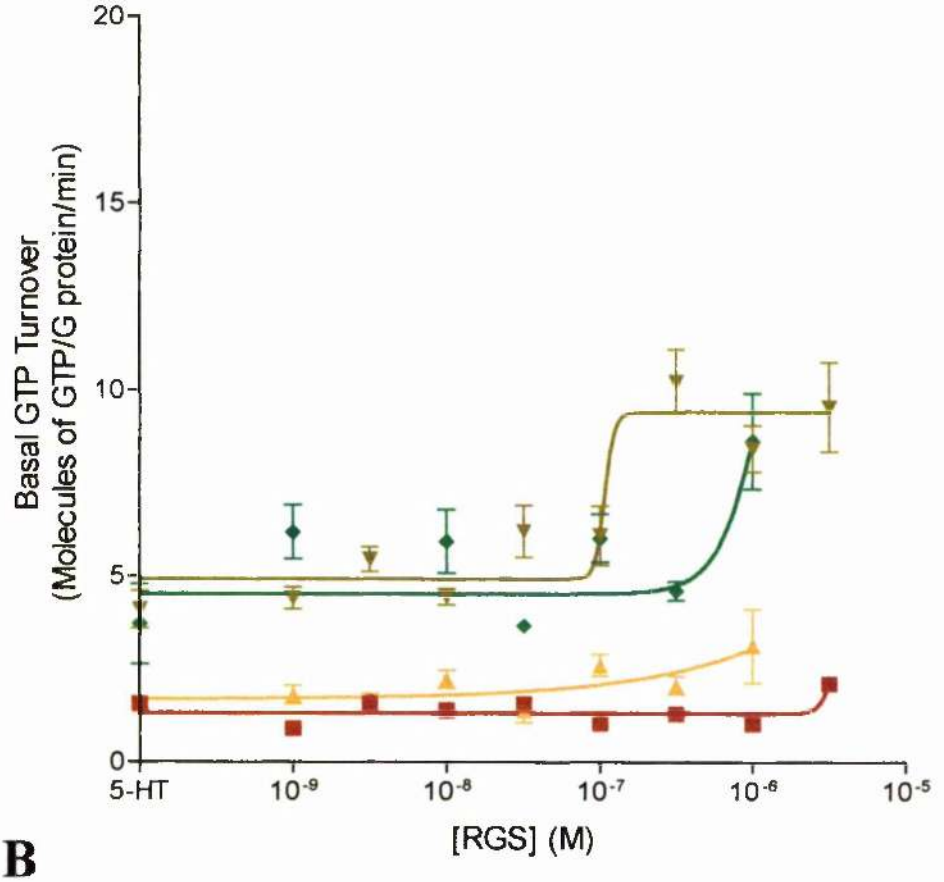
5-$HT_{1A}/G_{i1\alpha}C^{351}G$ + RGS1

5-$HT_{1A}/G_{i1\alpha}C^{351}G$ + RGS16

5-$HT_{1A}/G_{i1\alpha}C^{351}I$ + RGS1

5-$HT_{1A}/G_{i1\alpha}C^{351}I$ + RGS16

A


Basal GTP Turnover
(Molecules of GTP/G protein/min)
20
15
10
5
0
5-HT
10-9
10-8
10-7
10-6
10-5
[RGS] (M)

B

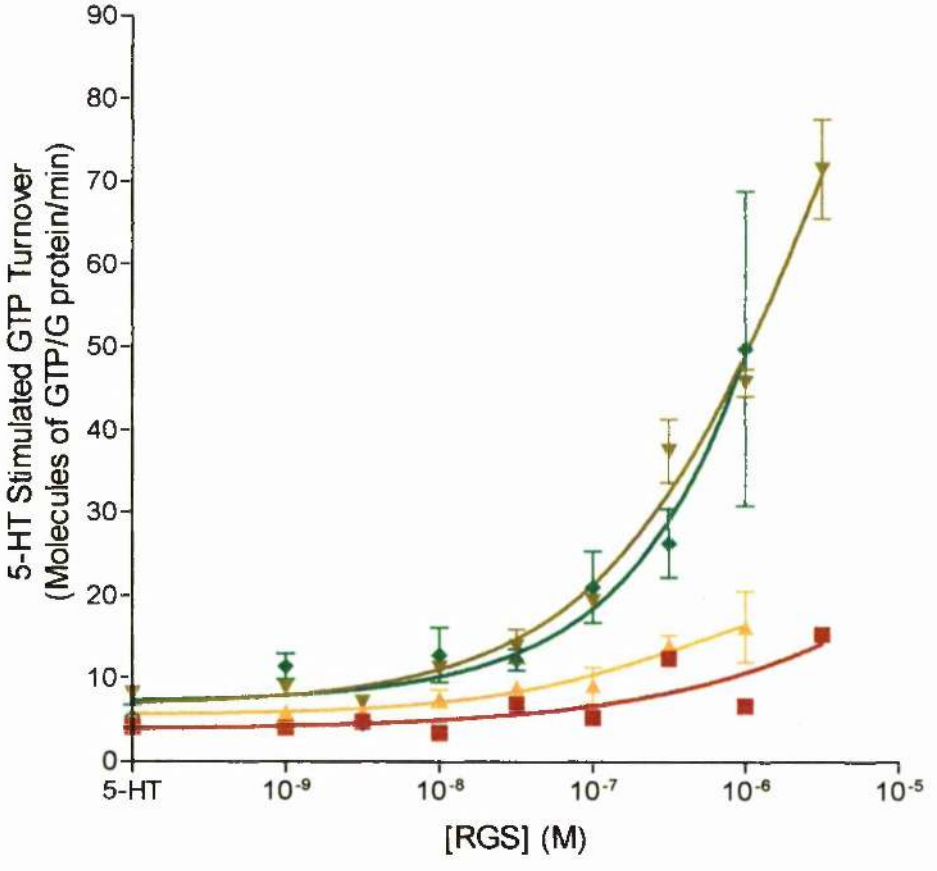

5-HT Stimulated GTP Turnover
(Molecules of GTP/G protein/min)
90
80
70
60
50
40
30
20
10
0
5-HT
10-9
10-8
10-7
10-6
10-5
[RGS] (M)

Figure 5.22 **RGS1 and RGS16 regulation of 5-HT stimulated GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}G/I$ fusion proteins**

The effects of increasing concentrations of RGS1 and RGS16 on 5-HT stimulated GTPase activity in membranes stably expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}G/I$ fusion proteins with the effects on basal GTP turnover removed.

Data are means of triplicate determinations and are representative of at least three similar experiments.

5-$HT_{1A}/G_{i1\alpha}C^{351}G$ + RGS1

5-$HT_{1A}/G_{i1\alpha}C^{351}G$ + RGS16

5-$HT_{1A}/G_{i1\alpha}C^{351}I$ + RGS1

5-$HT_{1A}/G_{i1\alpha}C^{351}I$ + RGS16

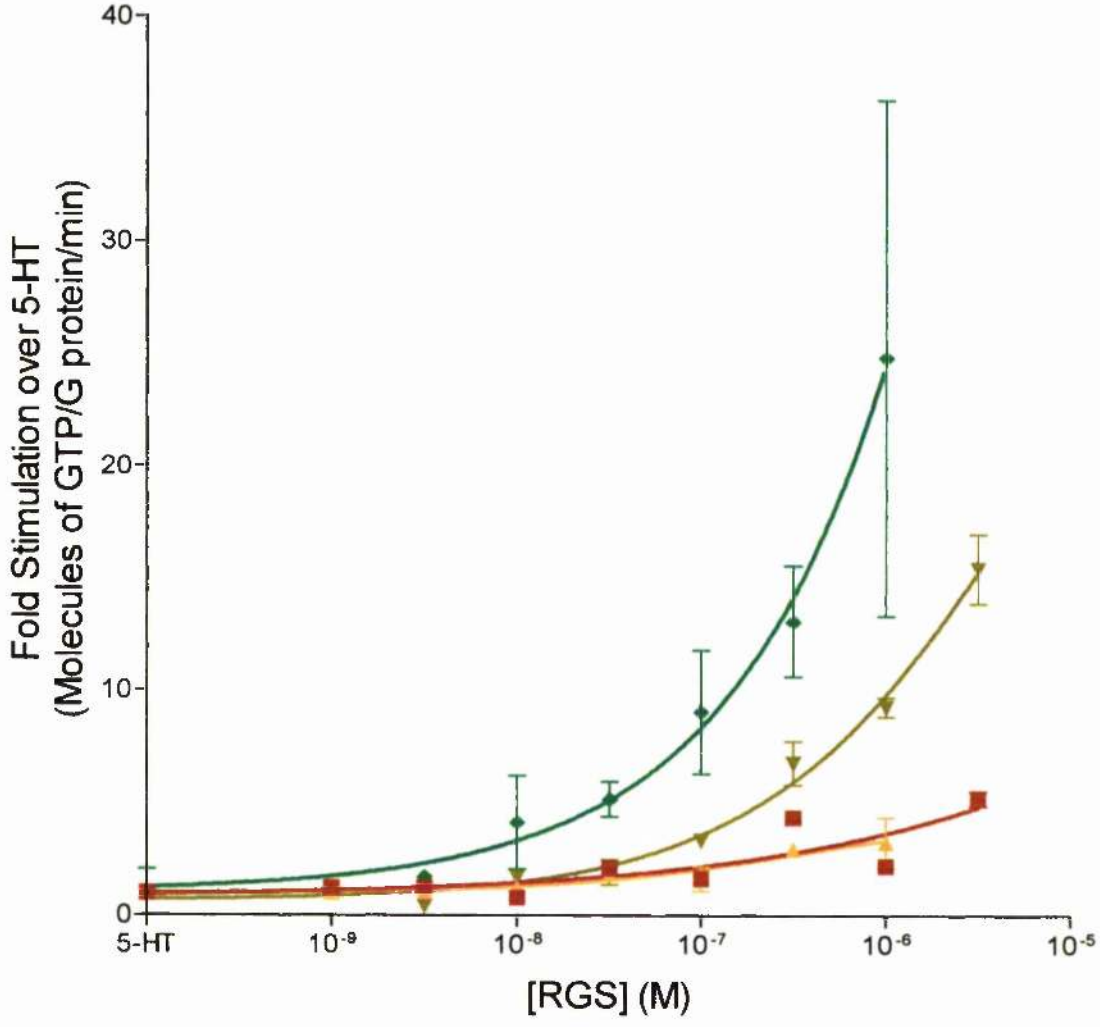
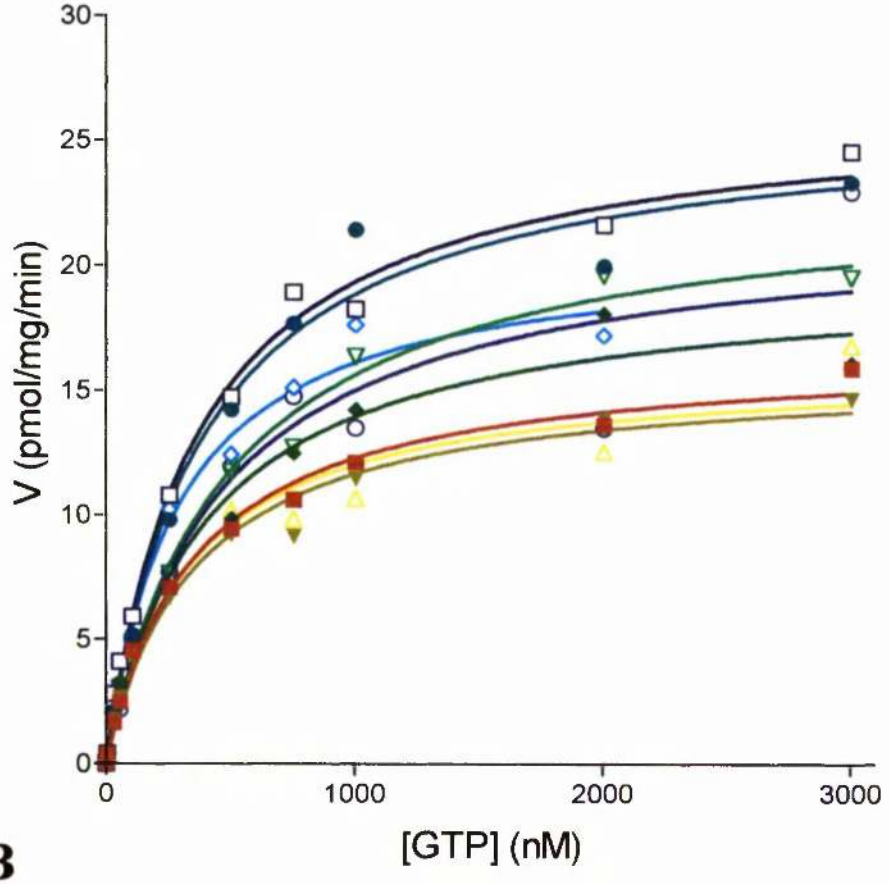

40
30
20
10
0
Fold Stimulation over 5-HT
(Molecules of GTP/G protein/min)
5-HT
10-9
10-8
10-7
10-6
10-5
[RGS] (M)

Figure 5.23 **Effects of increasing concentrations of RGS1 on the high affinity GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G$ fusion protein with increasing concentrations of GTP as substrate. Basal GTPase activity was measured in the presence of various concentrations of RGS1. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


30
25
20
15
10
5
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

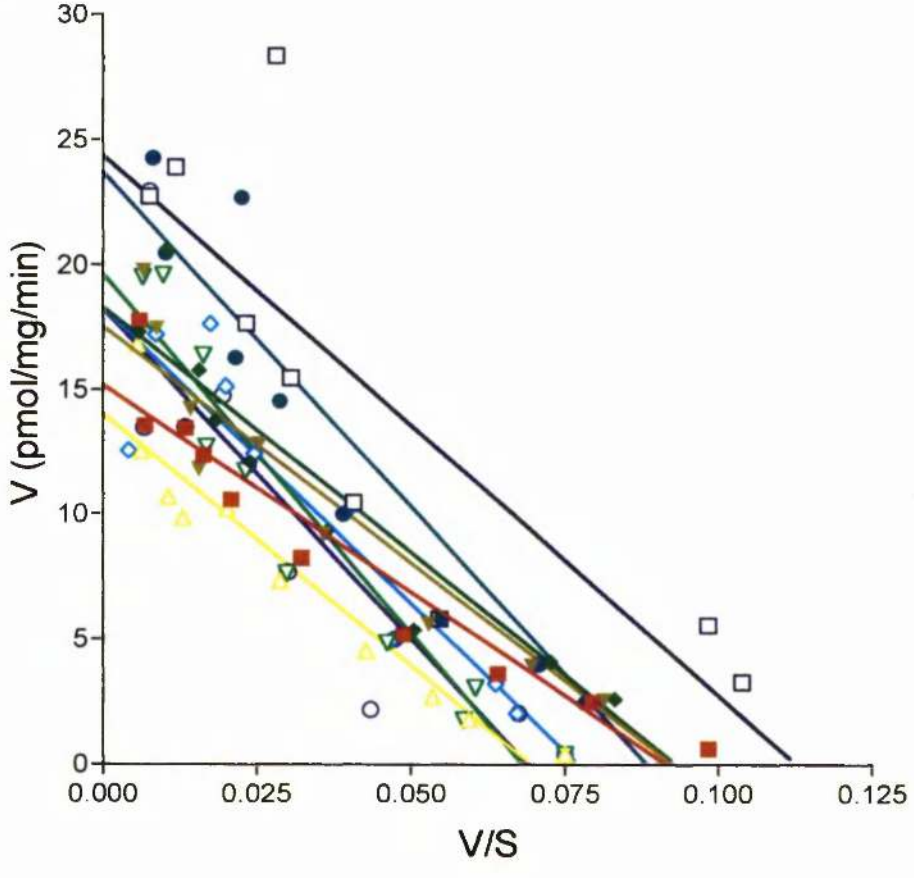

30
25
20
15
10
5
0
V (pmol/mg/min)
0.000
0.025
0.050
0.075
0.100
0.125
V/S

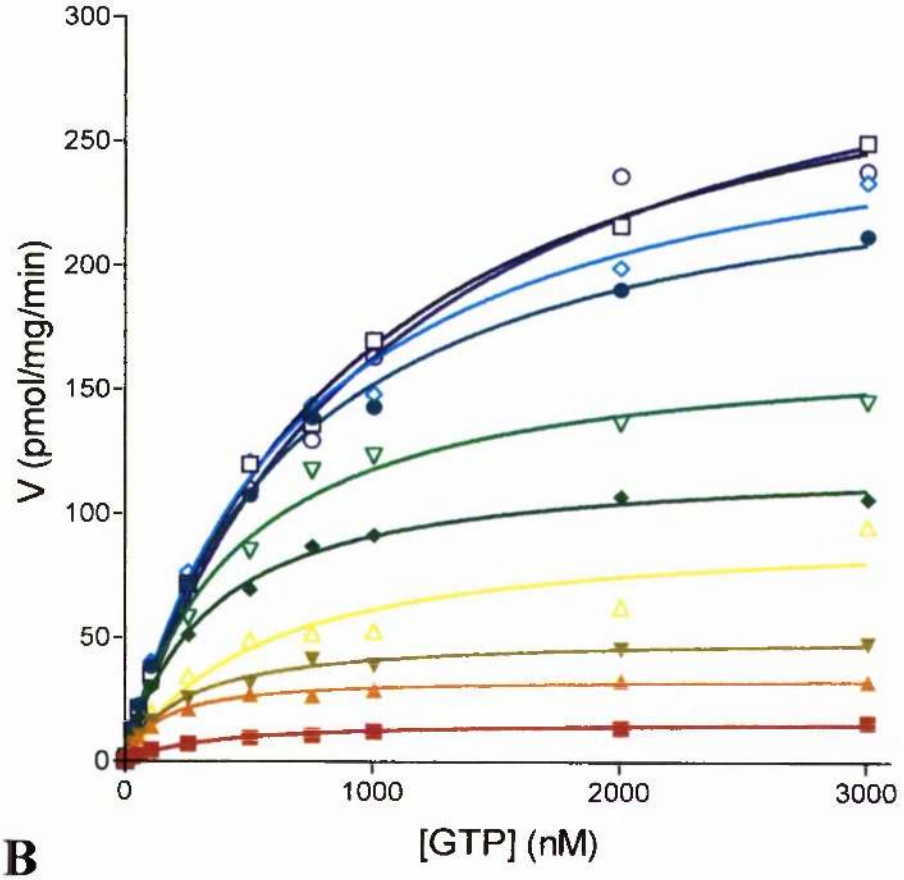
Figure 5.24 **Effects of increasing concentrations of RGS1 on the 5-HT stimulated GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G$ fusion protein with increasing concentrations of GTP as substrate. 5-HT stimulated GTPase activity was measured in the presence of various concentrations of RGS1. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

- Basal
- 5-HT
- 5-HT + RGS1 1nM
- 5-HT + RGS1 5nM
- 5-HT + RGS1 10nM
- 5-HT + RGS1 50nM
- 5-HT + RGS1 100nM
- 5-HT + RGS1 500nM
- 5-HT + RGS1 1μM
- 5-HT + RGS1 5μM

A


300
250
200
150
100
50
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

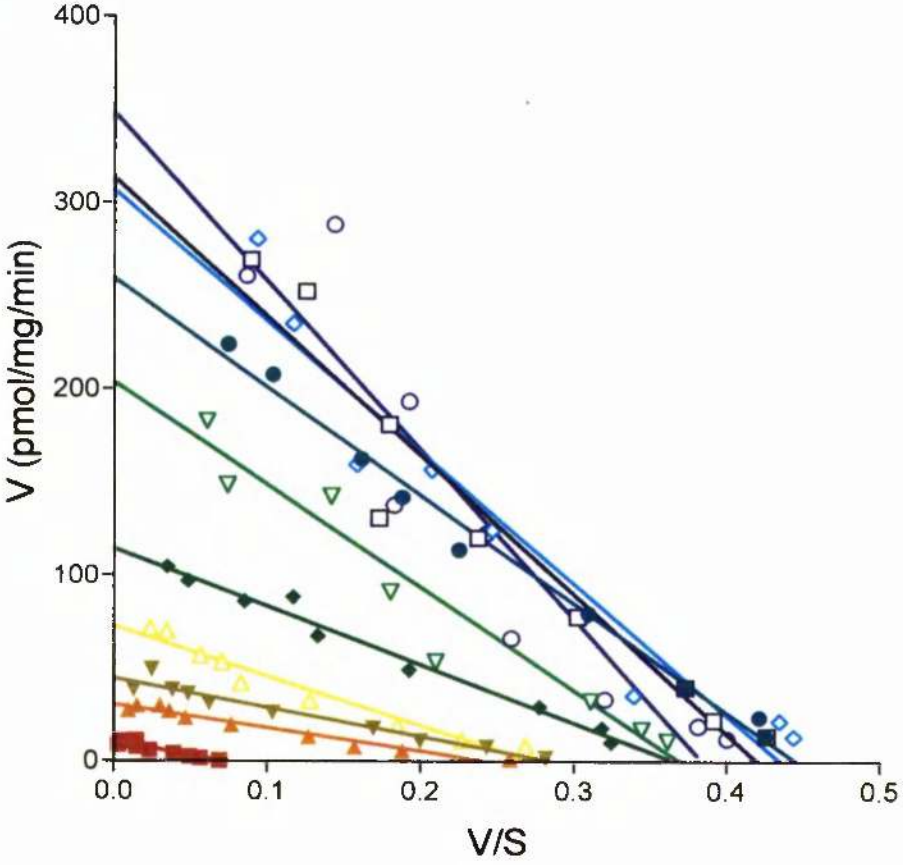
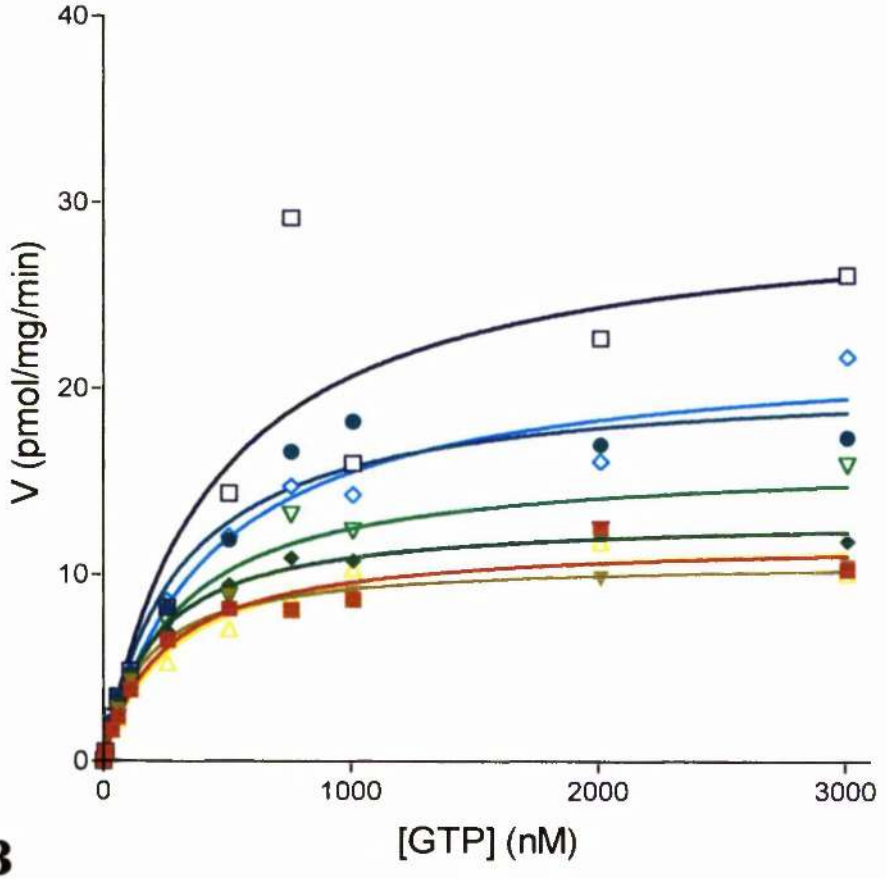

400
300
200
100
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
0.5
V/S

Figure 5.25 **Effects of increasing concentrations of RGS16 on the high affinity GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G$ fusion protein with increasing concentrations of GTP as substrate. Basal GTPase activity was measured in the presence of various concentrations of RGS16. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


40
30
20
10
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

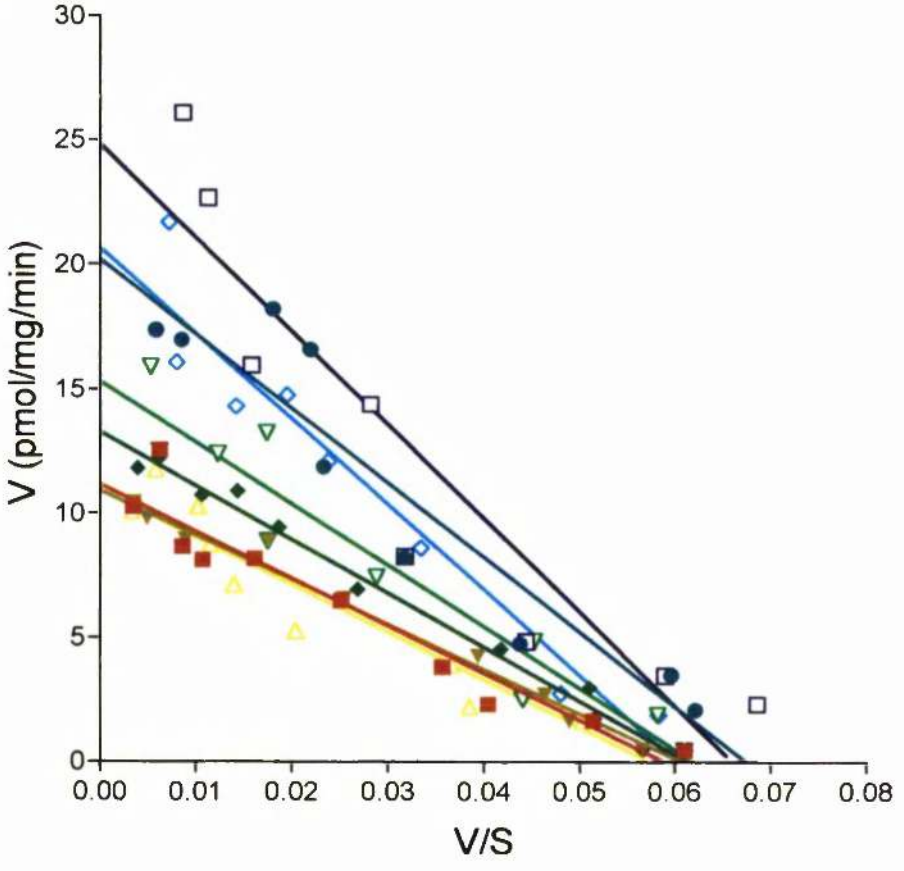
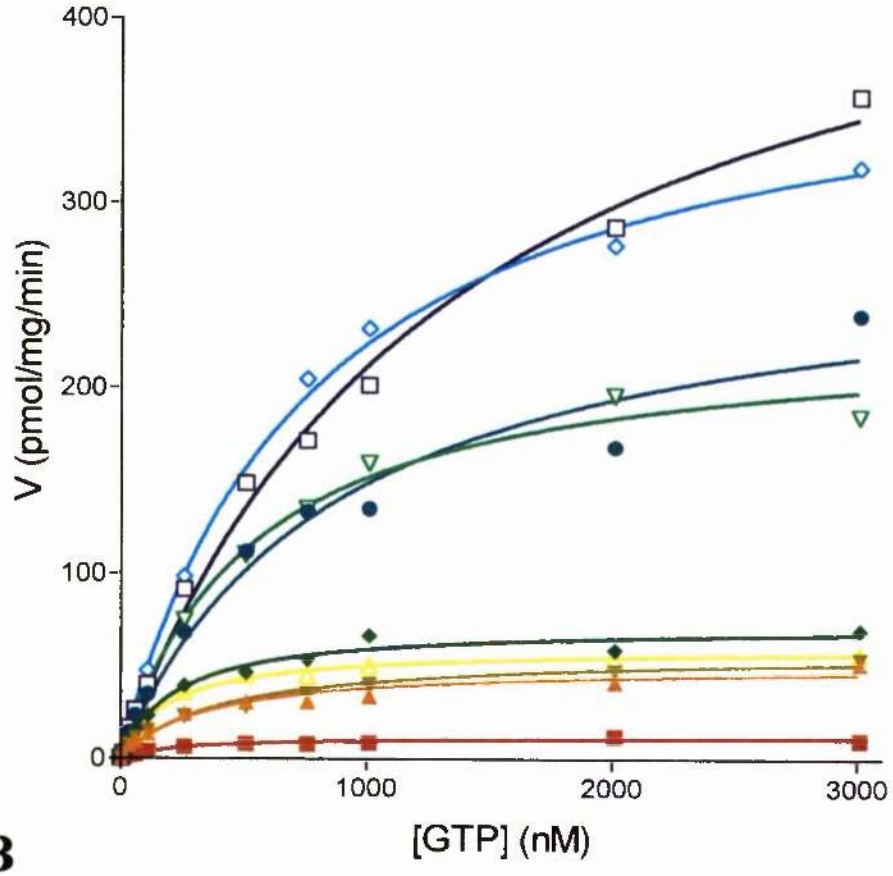

30
25
20
15
10
5
0
V (pmol/mg/min)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
V/S

Figure 5.26 **Effects of increasing concentrations of RGS16 on the 5-HT stimulated GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G$ fusion protein with increasing concentrations of GTP as substrate. 5-HT stimulated GTPase activity was measured in the presence of various concentrations of RGS16. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

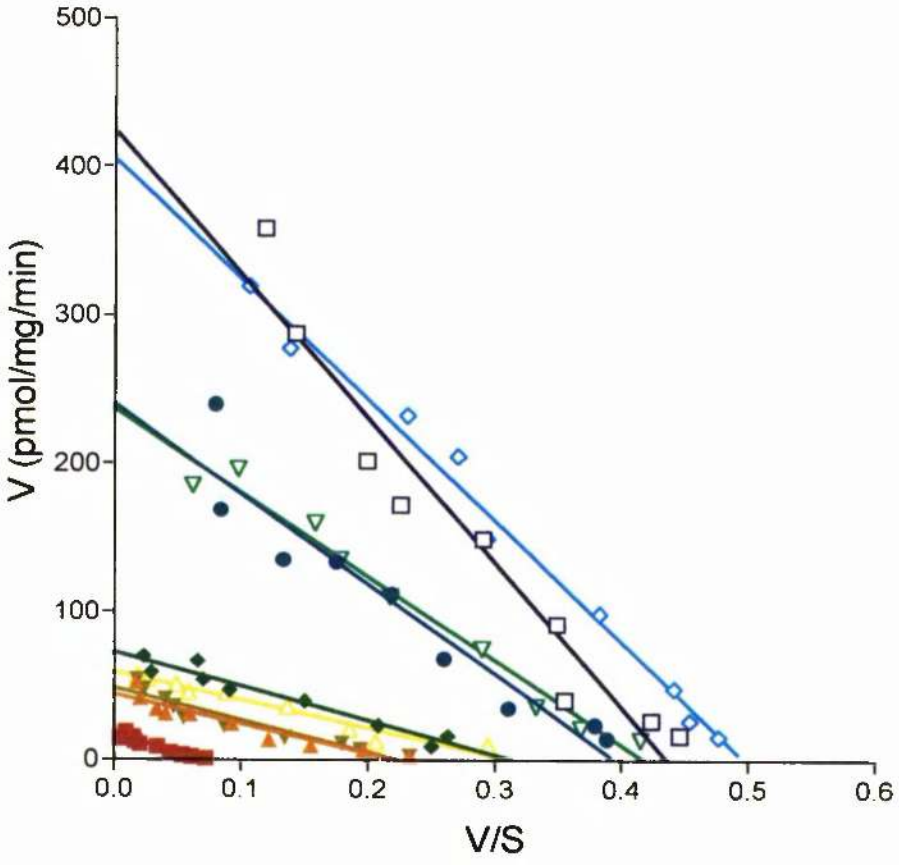

500
400
300
200
100
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
V/S

Table 5.5 **Mean K_m and V_{max} values for RGS protein concentration response high affinity GTPase assays with the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein**

Panel A: Effects of a range of RGS1 and RGS16 protein concentrations on the K_m and V_{max} of basal high affinity GTPase activity in membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein.

Panel B: Effects of a range of RGS1 and RGS16 protein concentrations on the K_m and V_{max} of 5-HT stimulated high affinity GTPase activity in membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein.

Data are mean +/- SEM from three similar experiments. An * denotes a result significantly different from the 0nM RGS protein concentration ($P < 0.05$). Values are in nM.

A

	RGS1		RGS16	
[RGS]	**Km**	**Vmax**	**Km**	**Vmax**
0nM (basal)	354+/-55	16.5+/-1.1	314+/-38	15.8+/-2.3
1nM	365+/-24	19.3+/-1.8	264+/-76	17.0+/-3.8
5nM	273+/-39	14.7+/-0.9	321+/-28	13.3+/-0.9
10nM	349+/-30	21.0+/-0.6	425+/-159	22.0+/-6.7
50nM	386+/-52	20.0+/-1.5	345+/-71	16.0+/-0.1
100nM	390+/-2	29.0+/-2.5	603+/-159	30.0+/-4.9
500nM	212+/-10	17.3+/-0.7	394+/-30	21.0+/-0.6
1μM	329+/-27	30.7+/-3.2*	395+/-40	27.7+/-1.2
5μM	337+/-61	18.3+/-2.0*		

$P < 0.05$

B

	RGS1		RGS16	
[RGS]	**Km**	**Vmax**	**Km**	**Vmax**
0nM (5-HT)	142+/-11	34.0+/-3.5	243+/-67	43.8+/-6.8
1nM+5-HT	279+/-68	66.7+/-18.2	345+/-71	60.0+/-8.7
5nM+5-HT	370+/-107	76.0+/-12.7	185+/-8	56.7+/-2.3*
10nM+5-HT	387+/-56	158.0+/-39.6	384+/-110	101.0+/-14.6*
50nM+5-HT	539+/-47*	179.0+/-17.8*	499+/-39*	220.7+/-7.9*
100nM+5-HT	784+/-102*	367.0+/-101.6*	833+/-80*	249.0+/-23.0*
500nM+5-HT	868+/-105*	290.3+/-42.5*	782+/-161*	374.0+/-50.1*
1μM +5-HT	908+/-123*	462.7+/-115.8*	1870+/-763*	532.0+/-128*
5μM +5-HT	991+/-105*	321.0+/-46.7*		

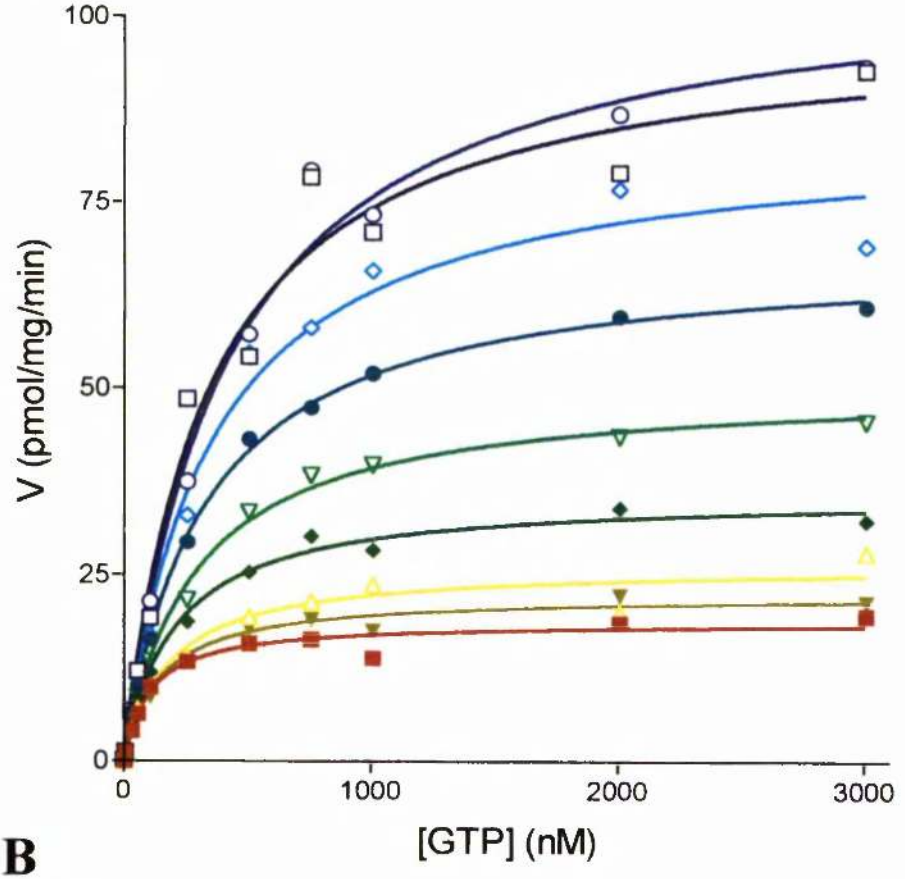
$P < 0.05$

Figure 5.27 **Effects of increasing concentrations of RGS1 on the high affinity GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. Basal GTPase activity was measured in the presence of various concentrations of RGS1. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


100
75
50
25
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

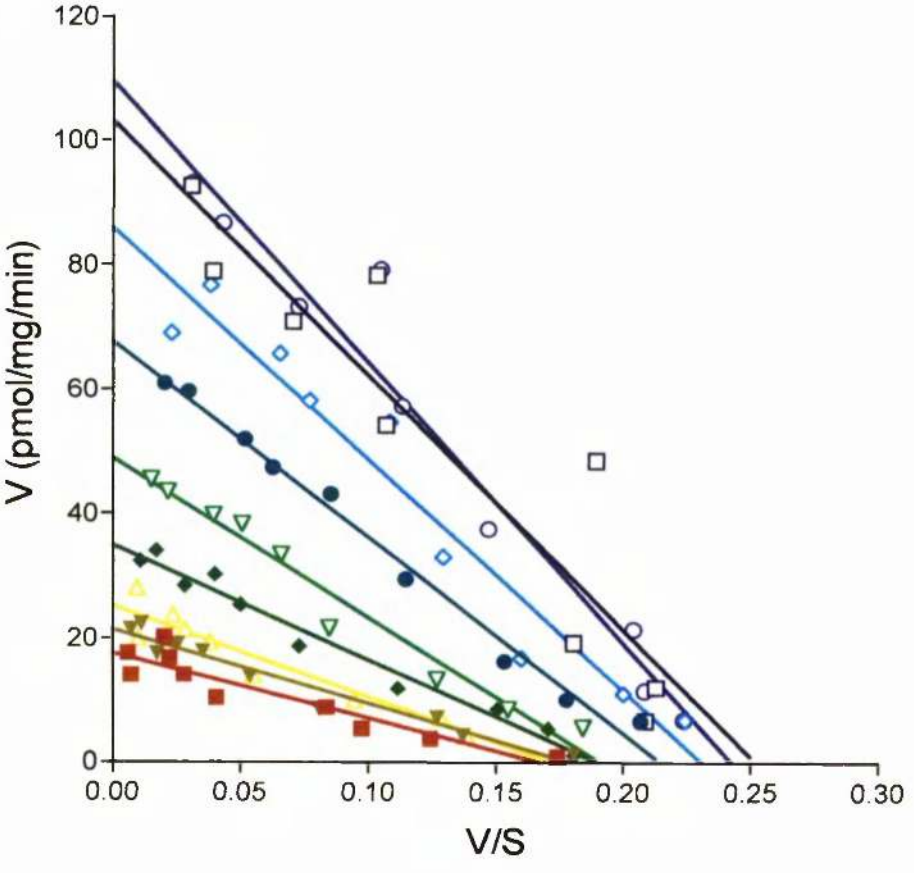

120
100
80
60
40
20
0
V (pmol/mg/min)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
V/S

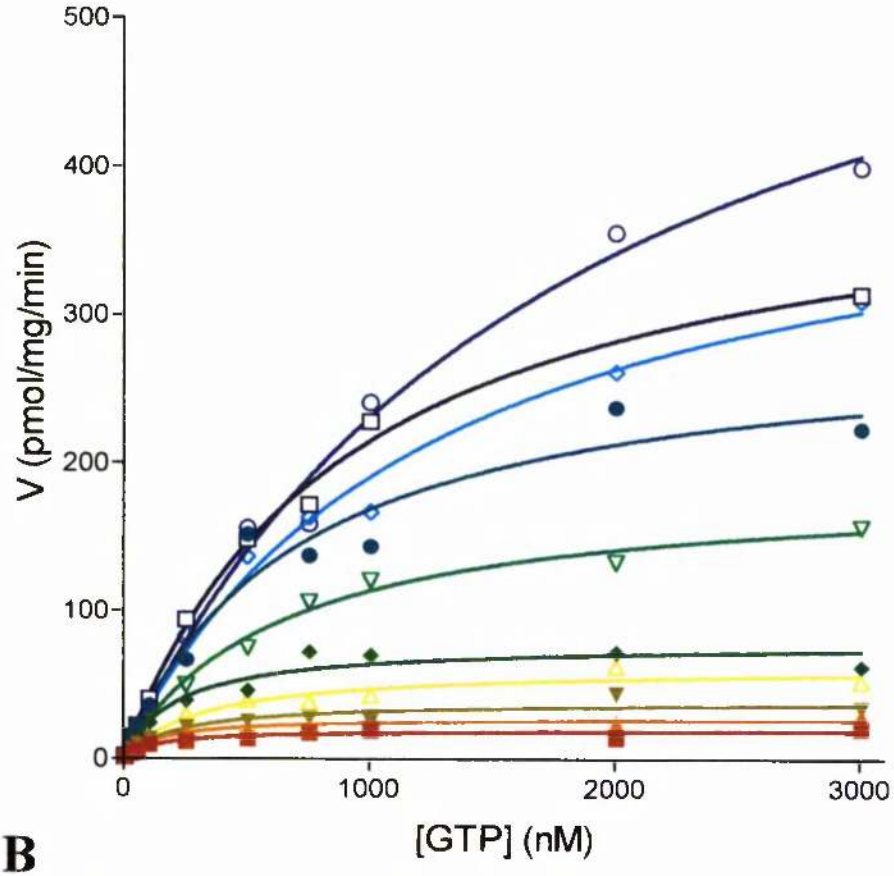
Figure 5.28 **Effects of increasing concentrations of RGS1 on the 5-HT stimulated GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. 5-HT stimulated GTPase activity was measured in the presence of various concentrations of RGS1. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

- Basal
- 5-HT
- 5-HT + RGS1 1nM
- 5-HT + RGS1 5nM
- 5-HT + RGS1 10nM
- 5-HT + RGS1 50nM
- 5-HT + RGS1 100nM
- 5-HT + RGS1 500nM
- 5-HT + RGS1 1μM
- 5-HT + RGS1 5μM

A


500
400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

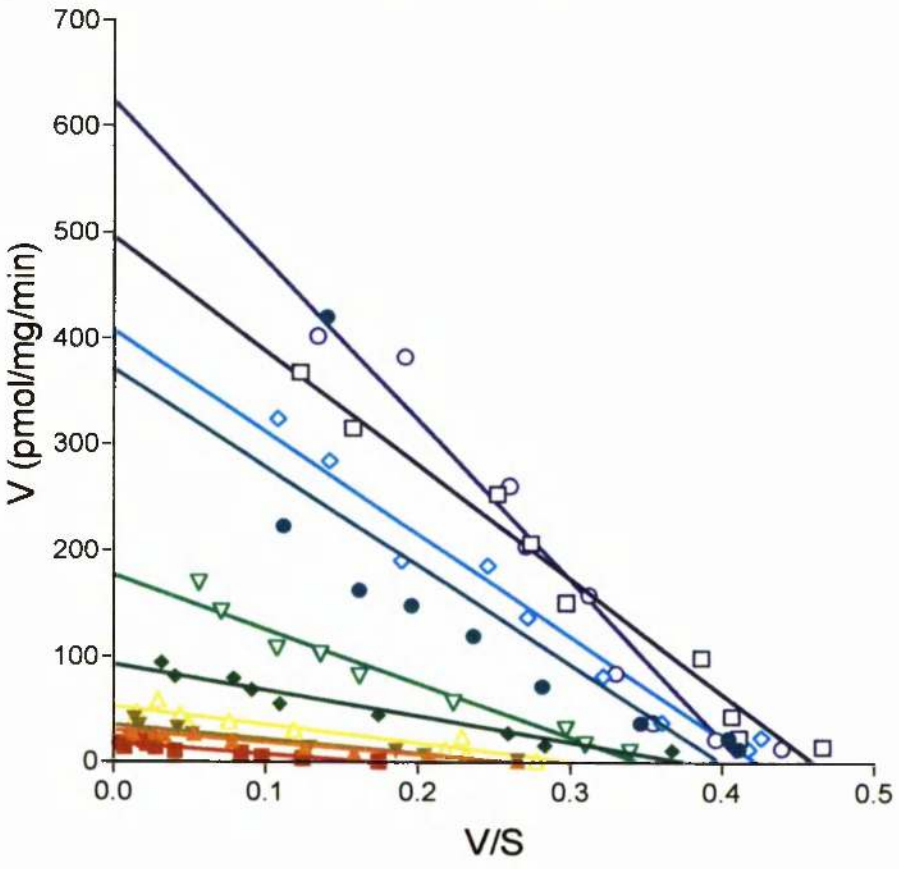
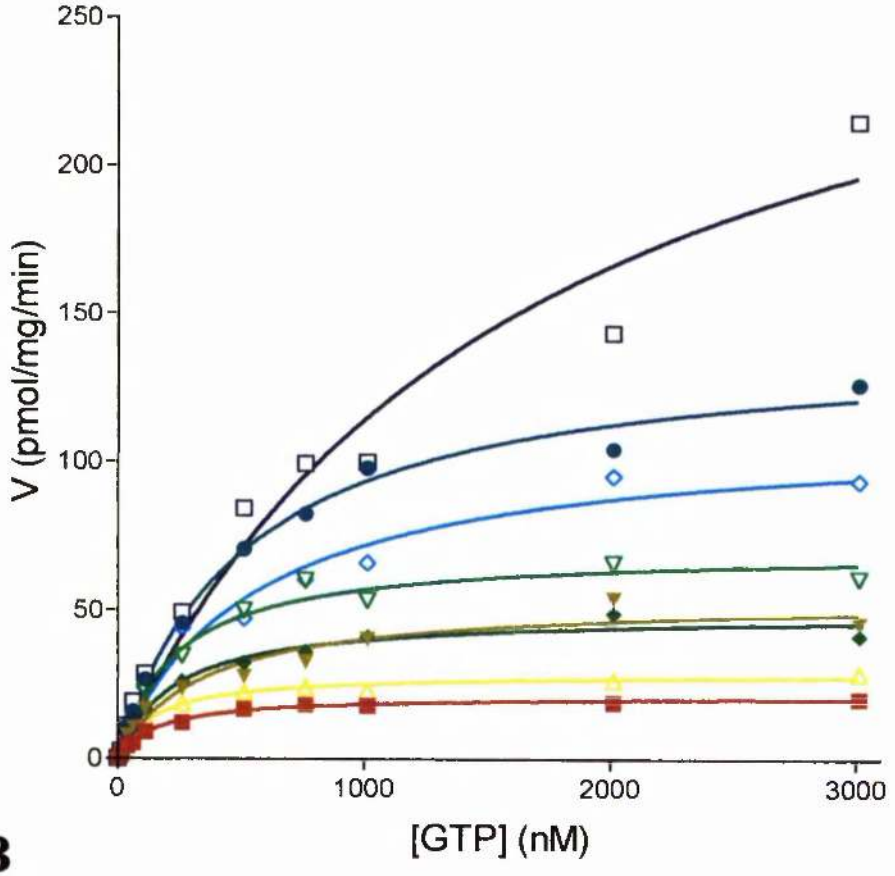

700
600
500
400
300
200
100
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
0.5
V/S

Figure 5.29 **Effects of increasing concentrations of RGS16 on the high affinity GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. Basal GTPase activity was measured in the presence of various concentrations of RGS16. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


250
200
150
100
50
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

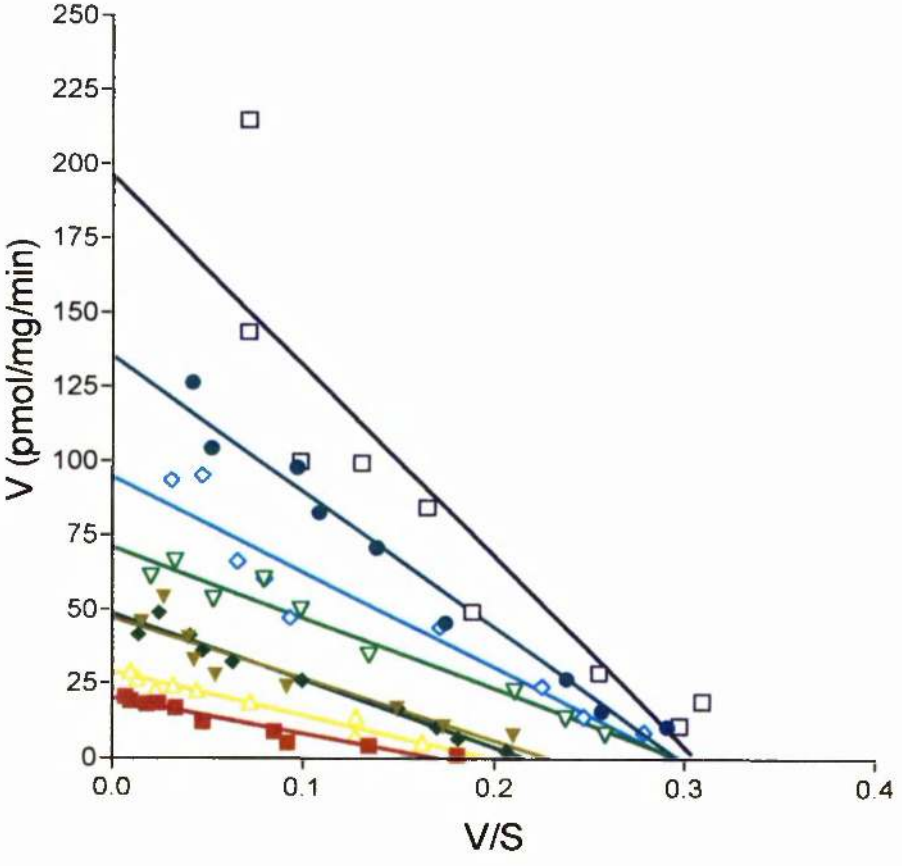

250
225
200
175
150
125
100
75
50
25
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
V/S

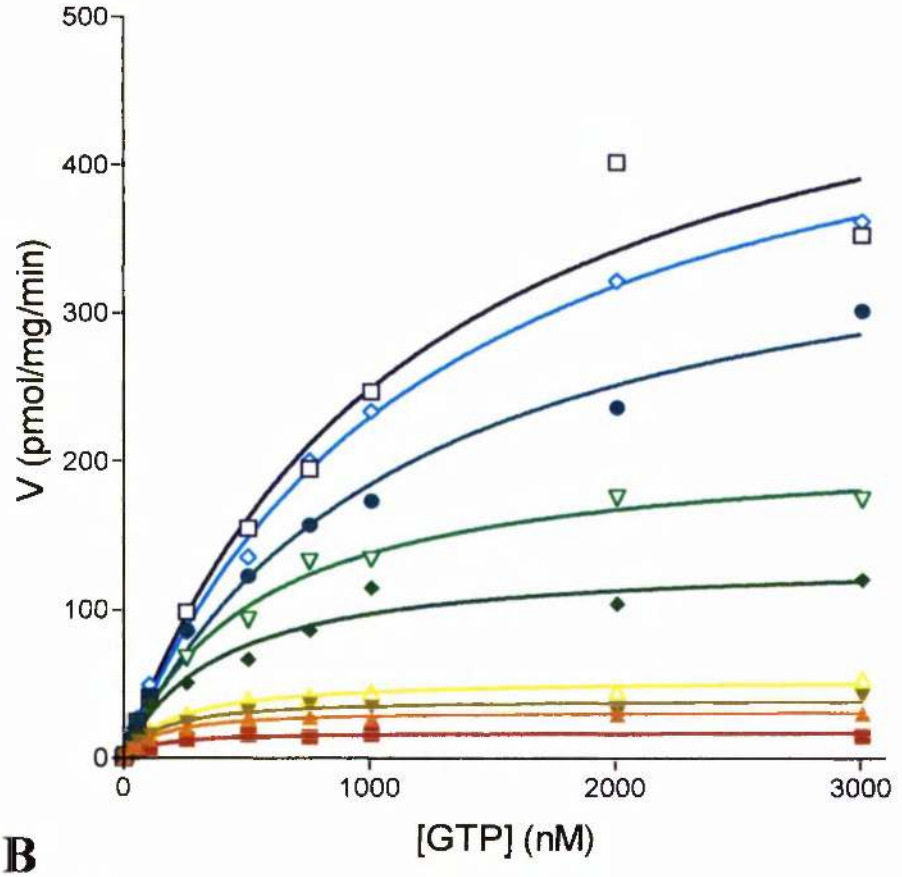
Figure 5.30 **Effects of increasing concentrations of RGS16 on the 5-HT stimulated GTPase activity of pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. 5-HT stimulated GTPase activity was measured in the presence of various concentrations of RGS16. Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

- Basal
- 5-HT
- 5-HT + RGS16 1nM
- 5-HT + RGS16 5nM
- 5-HT + RGS16 10nM
- 5-HT + RGS16 50nM
- 5-HT + RGS16 100nM
- 5-HT + RGS16 500nM
- 5-HT + RGS16 1μM

A


500
400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

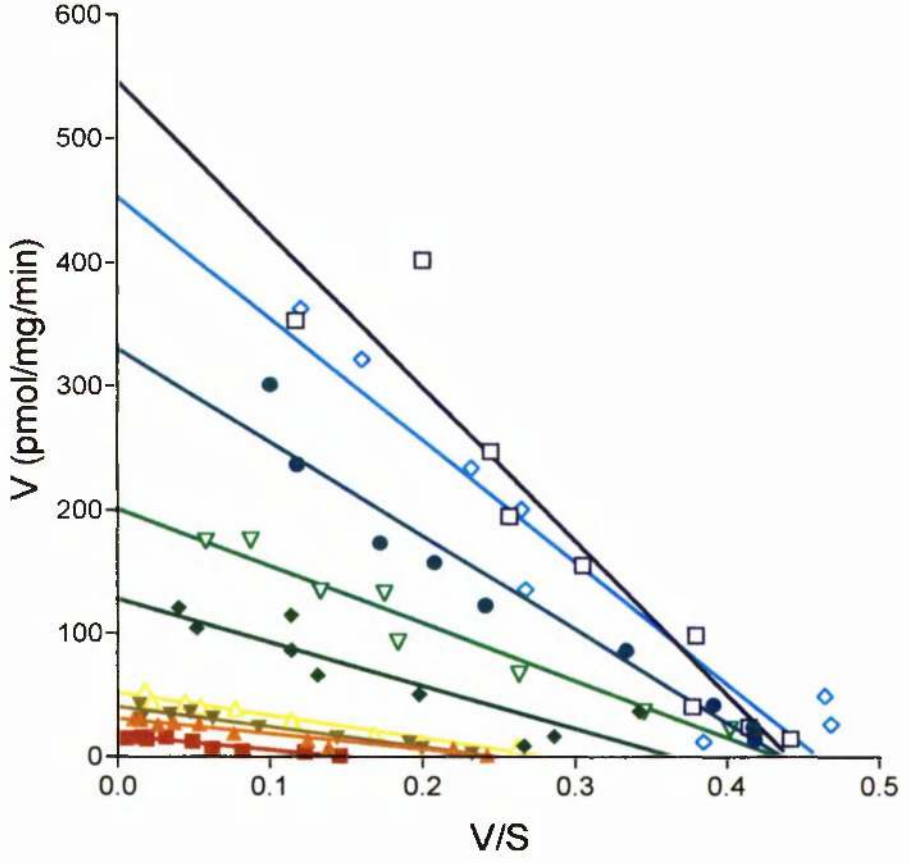

600
500
400
300
200
100
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
0.5
V/S

Table 5.6 **Mean K_m and V_{max} values for RGS protein concentration response high affinity GTPase assays with the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein**

Panel A: Effects of a range of RGS1 and RGS16 protein concentrations on the K_m and V_{max} of basal high affinity GTPase activity in membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.

Panel B: Effects of a range of RGS1 and RGS16 protein concentrations on the K_m and V_{max} of 5-HT stimulated high affinity GTPase activity in membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.

Data are mean +/- SEM from three similar experiments. An * denotes a result significantly different from the 0nM RGS protein concentration ($P < 0.05$). Values are in nM.

A

	RGS1		RGS16	
[RGS]	**Km**	**Vmax**	**Km**	**Vmax**
0nM (basal)	127+/-13	19.0+/-0.9	289+/-134	37.2+/-14.7
1nM	157+/-13	26.0+/-2.7	297+/-92	46.7+/-12.1
5nM	219+/-27	27.7+/-0.9	137+/-3	26.3+/-1.7
10nM	229+/-37	37.3+/-1.9*	349+/-139	69.3+/-26.6*
50nM	372+/-43	54.0+/-1.7*	290+/-33	80.7+/-7.1*
100nM	332+/-3*	78.7+/-6.9*	732+/-183	169.0+/-20.0*
500nM	393+/-28*	87.3+/-3.4*	410+/-62	108.0+/-2.5*
1μM	510+/-203*	123.3+/-15.8*	1389+/-181*	302.0+/-44.5*
5μM	392+/-15*	94.3+/-6.4*		

B

	RGS1		RGS16	
[RGS]	**Km**	**Vmax**	**Km**	**Vmax**
0nM (5-HT)	134+/-6	28.0+/-0.8	143+/-13	46.0+/-12.2
1nM+5-HT	210+/-11	39.0+/-1.0*	226+/-65	94.7+/-42.8
5nM+5-HT	236+/-48	53.3+/-5.2*	186+/-13*	47.7+/-3.8*
10nM+5-HT	323+/-54	90.3+/-6.5*	549+/-161*	171.7+/-42.3*
50nM+5-HT	675+/-25*	184.7+/-9.0*	610+/-37*	239.0+/-18.0*
100nM+5-HT	793+/-43*	294.0+/-9.6*	1191+/-87*	498.7+/-72.6*
500nM+5-HT	1097+/-110*	406.7+/-30.4*	1485+/-116*	588.7+/-49.9*
1μM +5-HT	1250+/-265*	530.3+/-81.4*	1824+/-318*	1030+/-242*
5μM +5-HT	1769+/-160*	632.3+/-12.9*		

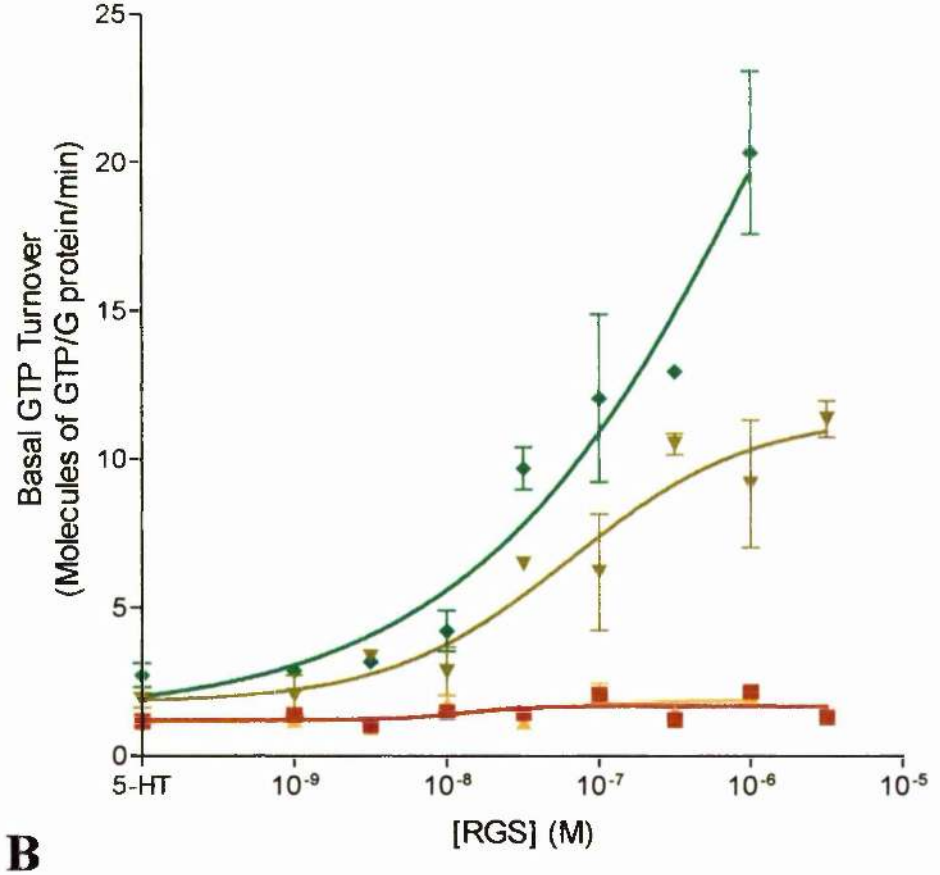
Figure 5.31 **RGS1 and RGS16 regulation of basal and 5-HT stimulated GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G/I$ fusion proteins**

Panel A: Utilising the V_{max} for GTP hydrolysis and B_{max} for expression levels, turnover numbers for basal GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}G/I$ fusion proteins in the presence of increasing concentrations of purified RGS1 and RGS16 were plotted. Panel B: Turnover numbers were plotted for 5-HT stimulated GTPase activity in the presence of various concentrations of RGS1 and 16.

Data are means of triplicate determinations and are representative of at least three similar experiments.

- 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ + RGS1
- 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ + RGS16
- 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ + RGS1
- 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ + RGS16

A


Basal GTP Turnover
(Molecules of GTP/G protein/min)
25
20
15
10
5
0
5-HT
10-9
10-8
10-7
10-6
10-5
[RGS] (M)

B

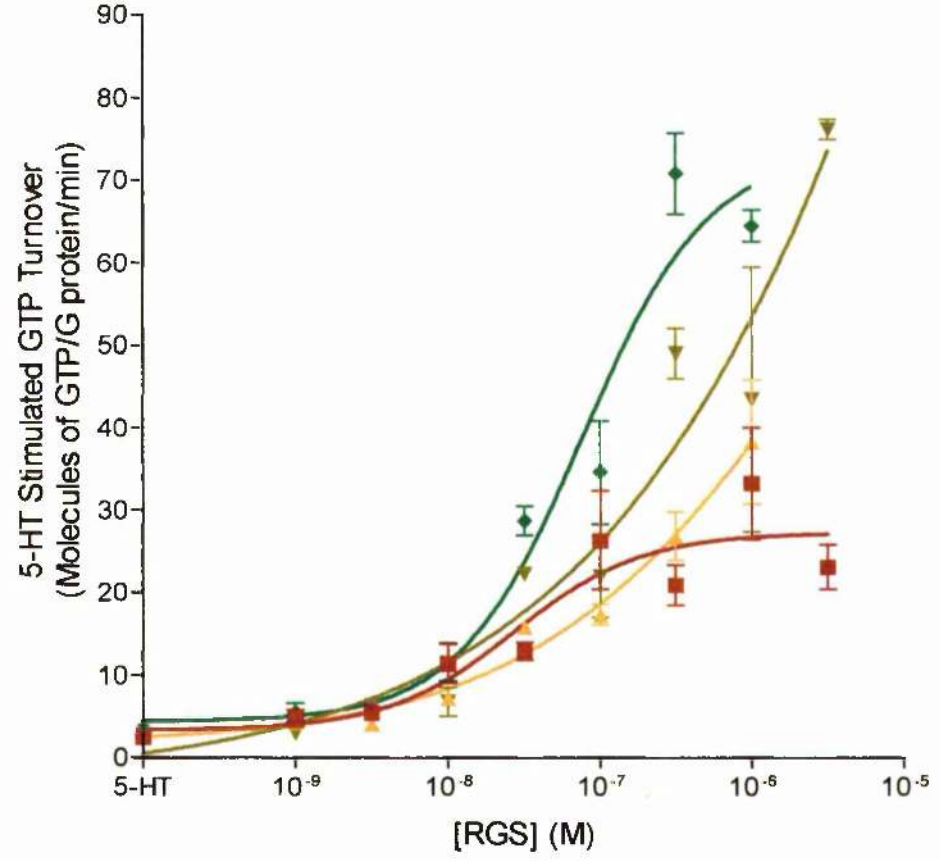

5-HT Stimulated GTP Turnover
(Molecules of GTP/G protein/min)
90
80
70
60
50
40
30
20
10
0
5-HT
10-9
10-8
10-7
10-6
10-5
[RGS] (M)

Figure 5.32 **RGS1 and RGS16 regulation of 5-HT stimulated GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G/I$ fusion proteins**

The effects of increasing concentrations of RGS1 and RGS16 on 5-HT stimulated GTPase activity in membranes stably expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G/I$ fusion proteins with the effects on basal GTP turnover removed.

Data are means of triplicate determinations and are representative of at least three similar experiments.

□ 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ + RGS1

△ 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ + RGS16

▽ 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ + RGS1

◆ 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ + RGS16

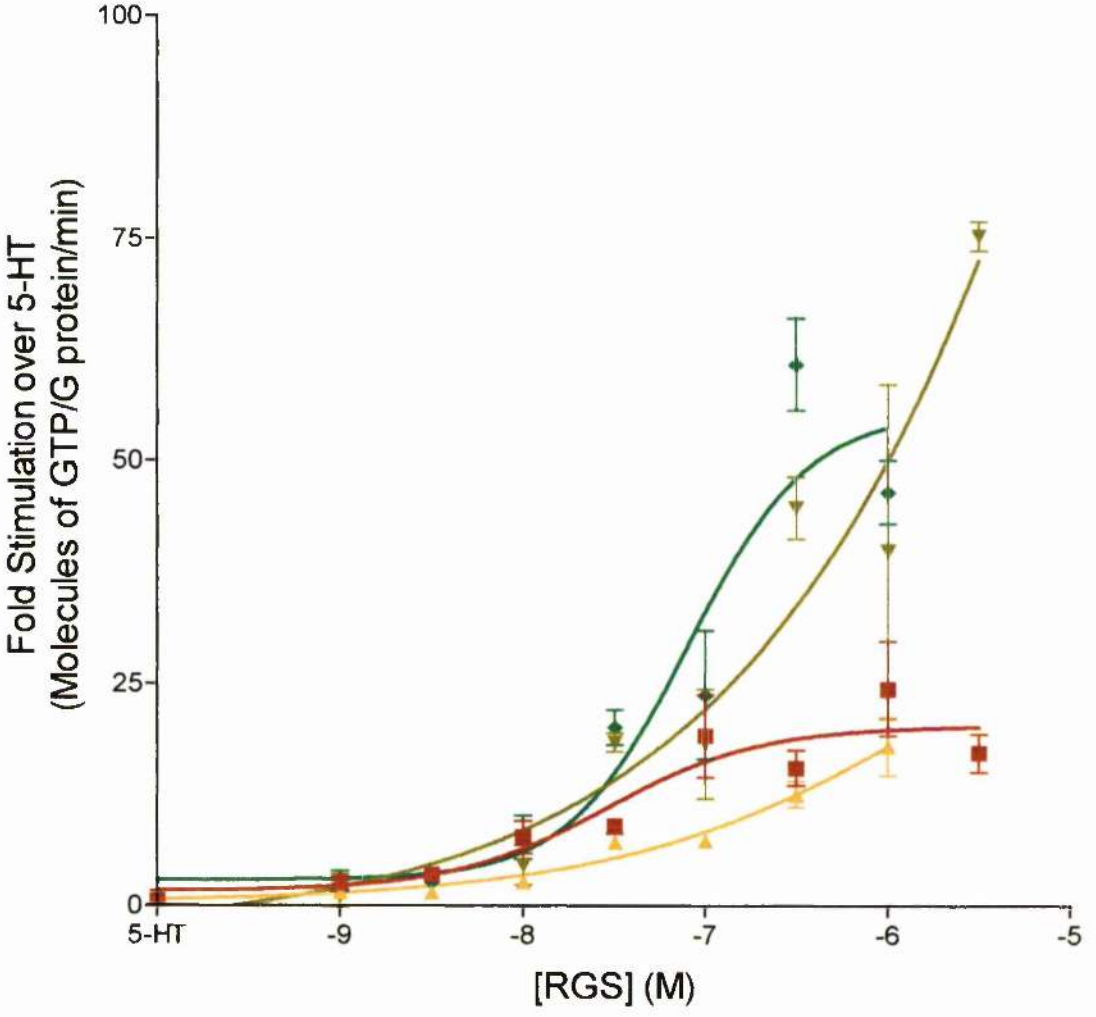

100
75
50
25
0
Fold Stimulation over 5-HT
(Molecules of GTP/G protein/min)
5-HT
-9
-8
-7
-6
-5
[RGS] (M)

Figure 5.33 Assays to examine the effects of endogenous HEK293 RGS proteins

Panel A: Basal and 5-HT (100μM) stimulated high affinity GTPase activity of membranes expressing the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion protein with RGS1 (10μM) and two cytosolic fractions normally discarded during membrane preparation.

- Basal
- 5-HT
- RGS1 (1μM)
- 5-HT + RGS1
- Cytosol A
- 5-HT + Cytosol A
- Cytosol B
- 5-HT + Cytosol B

Panel B: Basal and 5-HT (100μM) stimulated high affinity GTPase activity in membranes expressing the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion protein, a sample of which had been pre-treated with 5-HT (100μM) for 10 minutes prior to cell harvesting.

- Basal
- 5-HT
- RGS1 (1μM)
- 5-HT + RGS1

Data are means of triplicate determinations and are representative of at least three similar experiments.

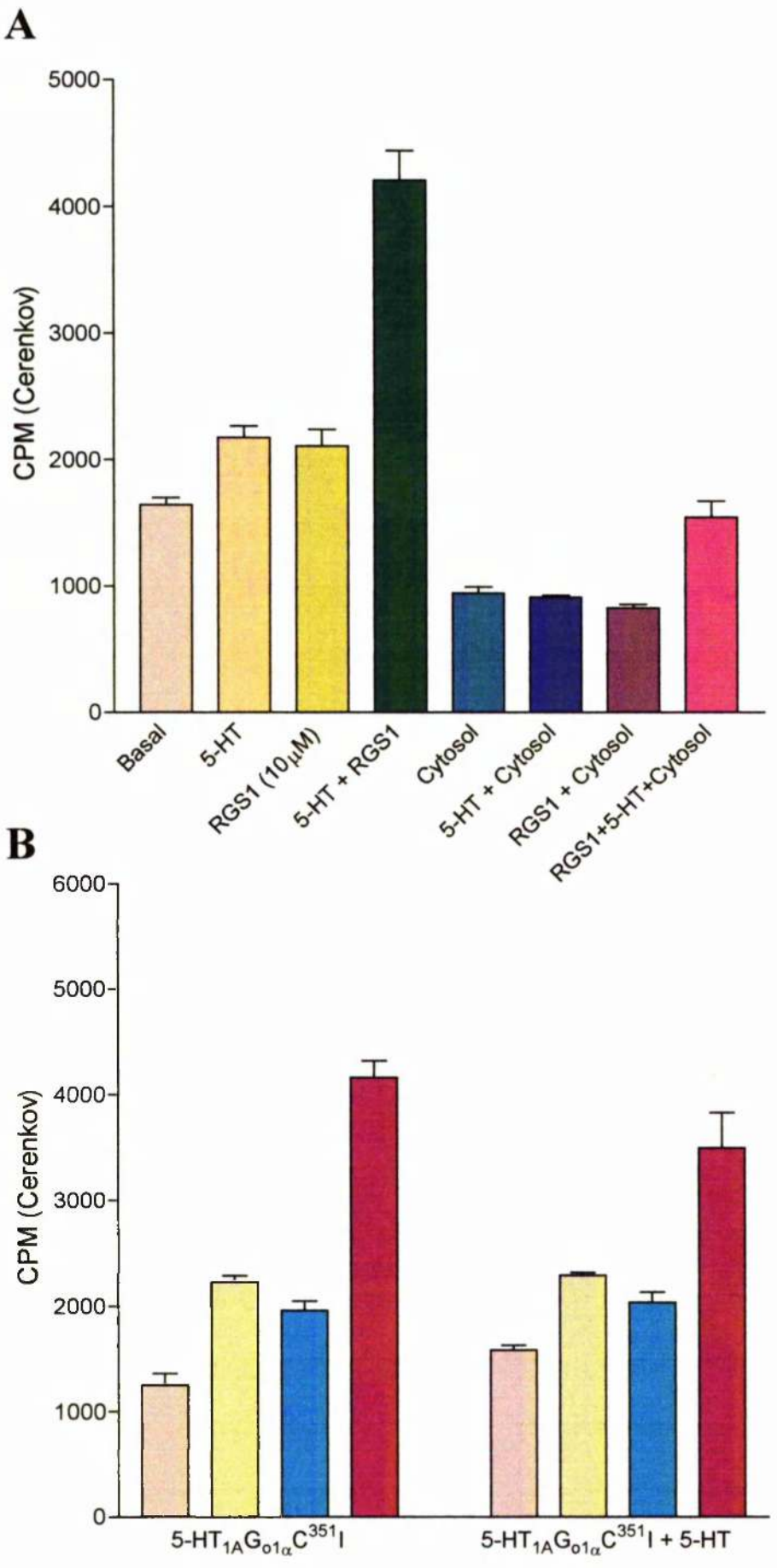

A
5000
4000
3000
2000
1000
0
CPM (Cerenkov)
Basal
5-HT
RGS1 (10μM)
5-HT + RGS1
Cytosol
5-HT + Cytosol
RGS1 + Cytosol
RGS1+5-HT+Cytosol
B
6000
5000
4000
3000
2000
1000
0
CPM (Cerenkov)
5-HT1AGo1αC351I
5-HT1AGo1αC351I + 5-HT

Table 5.7 Table of RGS proteins reported to be expressed either ubiquitously or in the human kidney

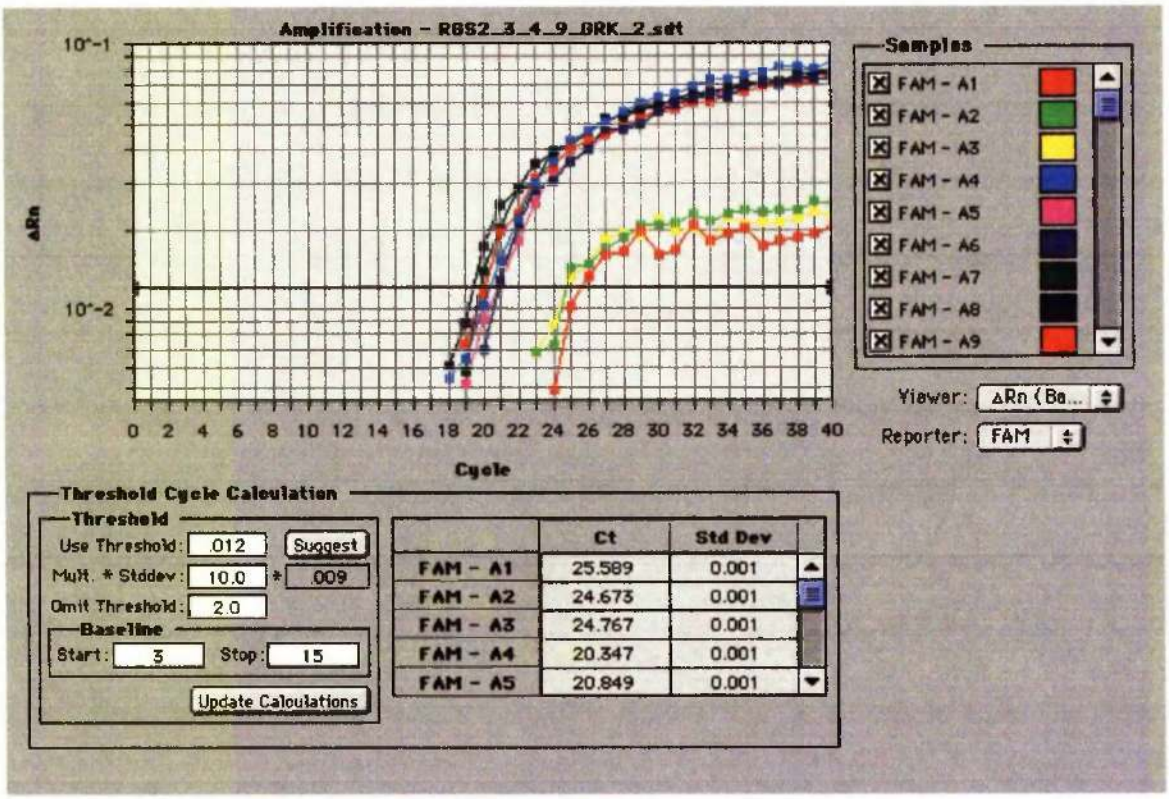
The table shows the RGS proteins of different classes reported in the literature as being expressed either ubiquitously or in the human kidney. Primers and probes for Taqman analysis of expression in HEK293 cells were designed for RGS2, RGS3, RGS4, RGS9 and GRK2.

RGS Protein	Tissue Expression
RGS GAIP, RGS2, RGS3, RGS4, RGS4 and RGS16	Ubiquitous
RGS9	Retina, lower levels in kidney
GRK2, GRK3, GRK5 and GRK6	Ubiquitous
Axin 1, p115RhoGEF and PDZRhoGEF	Ubiquitous

Figure 5.34 PCR amplification of RGS2 and GRK2 cDNA using primers designed for Taqman QrtPCR

PCR conditions were established for RGS2 (Panel A) and GRK2 (Panel B) using cDNA samples prior to running the Taqman experiment on genomic HEK293 DNA. Six concentrations of cDNA were utilised (10ng/μl, 1ng/μl, 100pg/μl, 10pg/μl, 1pg/μl and 100fg/μl) at three different primer concentrations (50:50, 300:300 and 900:900 (nM) forward: reverse) for forty cycles. Data are plotted as increase in absorbance against cycle number. Data are means of triplicate determinations and are representative of at least three similar experiments.

A


Amplification - RGS2_3_4_9_BRK_2.sdt
10^-1
10^-2
ΔRn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
Samples
FAM - A1
FAM - A2
FAM - A3
FAM - A4
FAM - A5
FAM - A6
FAM - A7
FAM - A8
FAM - A9
Viewer: ΔRn (Ba...
Reporter: FAM
Threshold Cycle Calculation
Threshold
Use Threshold: .012
Suggest
Mult. * Stddev: 10.0 * .009
Omit Threshold: 2.0
Baseline
Start: 3 Stop: 15
Update Calculations
Ct
Std Dev
FAM - A1 25.589 0.001
FAM - A2 24.673 0.001
FAM - A3 24.767 0.001
FAM - A4 20.347 0.001
FAM - A5 20.849 0.001

B

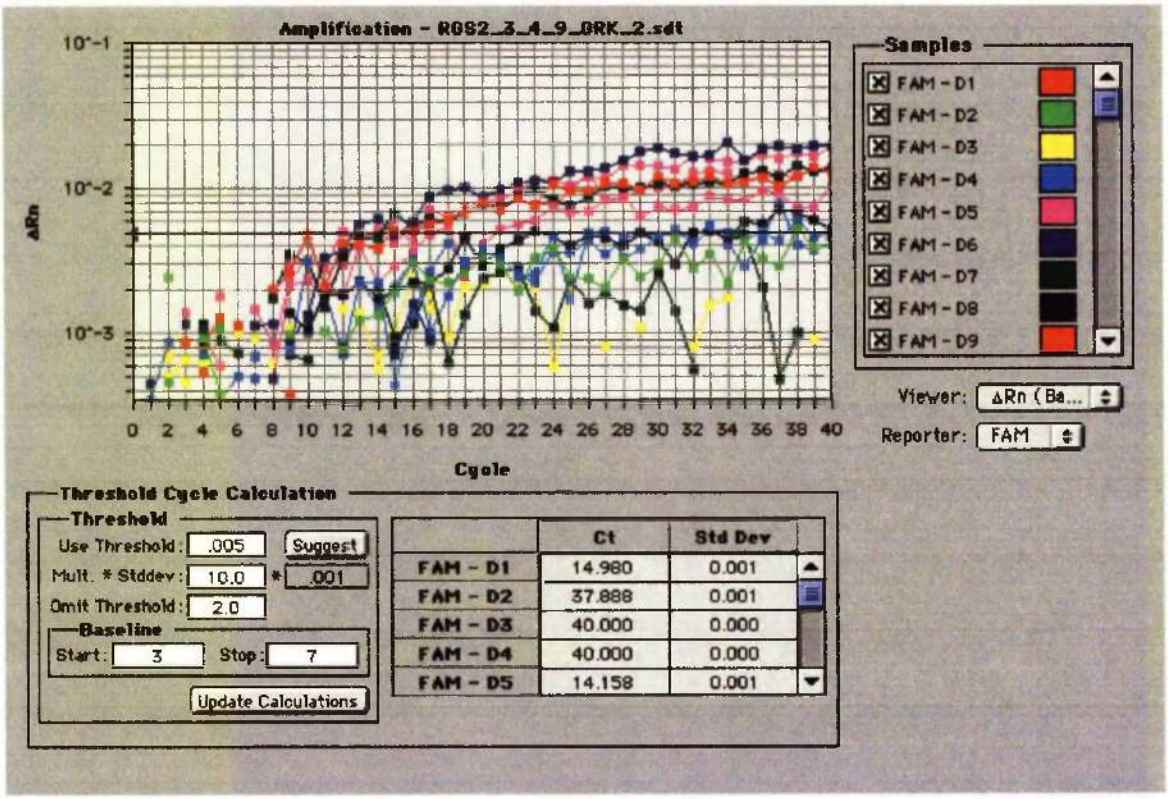
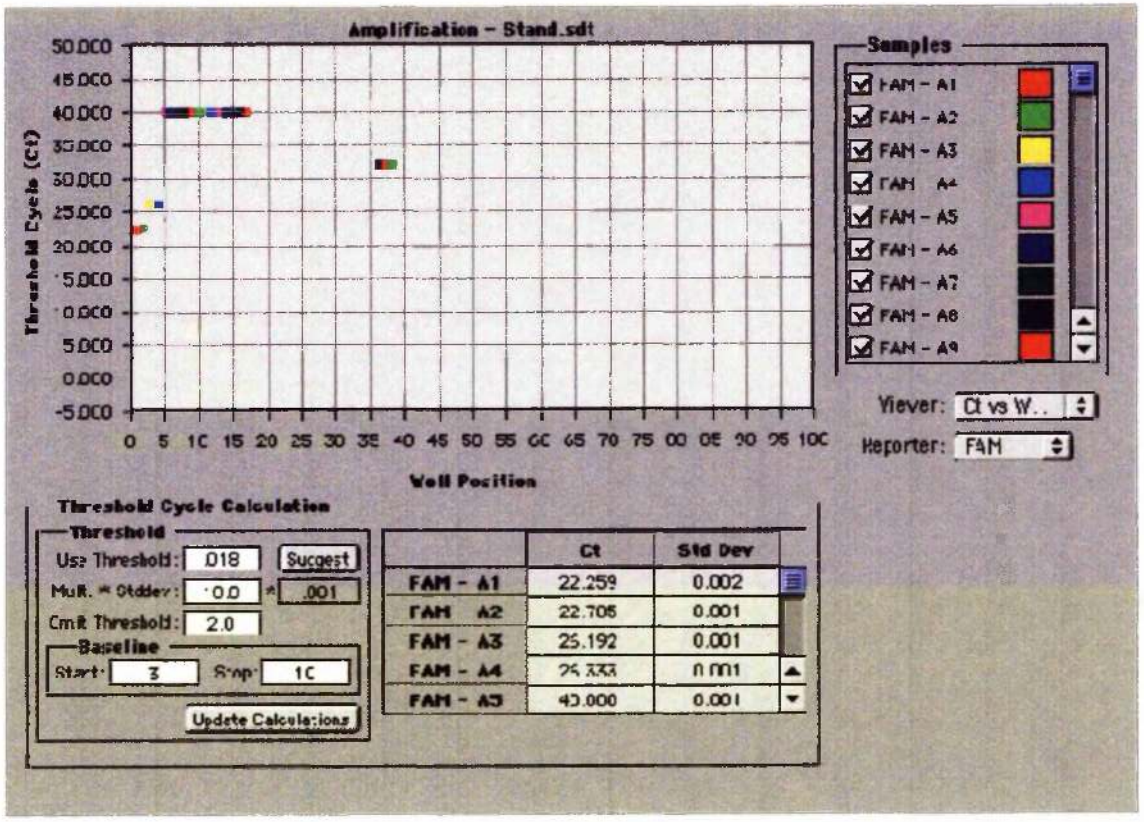

Amplification - RGS2_3_4_9_BRK_2.sdt
10^-1
10^-2
10^-3
ΔRn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle
Samples
FAM - D1
FAM - D2
FAM - D3
FAM - D4
FAM - D5
FAM - D6
FAM - D7
FAM - D8
FAM - D9
Viewer: ΔRn (Ba...
Reporter: FAM
Threshold Cycle Calculation
Threshold
Use Threshold: .005
Suggest
Mult. * Stddev: 10.0 * .001
Omit Threshold: 2.0
Baseline
Start: 3 Stop: 7
Update Calculations
Ct
Std Dev
FAM - D1 14.980 0.001
FAM - D2 37.888 0.001
FAM - D3 40.000 0.000
FAM - D4 40.000 0.000
FAM - D5 14.158 0.001

Figure 5.35 PCR amplification of RGS2 from genomic HEK293 DNA using Taqman QrtPCR

Using previously established PCR conditions for RGS2 cDNA an experiment on genomic HEK293 DNA was completed. Well position 0-20 are the results of the standard curve using 10ng/µl of RGS2 cDNA per well at a primer ratio of 300:300nM. Well positions 36-38 are the results for the genomic HEK293 DNA. Data are means of triplicate determinations.


Amplification - Stand.sdt
Threshold Cycle (Ct)
50.000
45.000
40.000
35.000
30.000
25.000
20.000
15.000
10.000
5.000
0.000
-5.000
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Well Position
Samples
FAM - A1
FAM - A2
FAM - A3
FAM - A4
FAM - A5
FAM - A6
FAM - A7
FAM - A8
FAM - A9
Viewer: Ct vs W..
Reporter: FAM
Threshold Cycle Calculation
Threshold
Use Threshold: .018
Suggest
Mult. * Stddev: 10.0 * .001
Cmit Threshold: 2.0
Baseline
Start: 3
Stop: 10
Update Calculations
Ct
Std Dev
FAM - A1 22.259 0.002
FAM - A2 22.705 0.001
FAM - A3 25.192 0.001
FAM - A4 25.333 0.001
FAM - A5 40.000 0.001

5.3 Discussion

High affinity GTPase assays measure the rate of GTP hydrolysis to GDP in a sample (McKenzie and Milligan, 1990; Wise *et al.*, 1997a,b). This is a particularly useful measure of the ability of ligands of GPCRs to alter the GTPase activity of the G protein to which they are coupled. For receptor-G protein fusion proteins incorporating a G_i-family G protein this is best taken advantage of by introducing a mutation at Cys^{351}, four amino acids from the end of the C terminal tail. This introduces an amino acid that cannot be ADP ribosylated by pertussis toxin. Pertussis toxin catalyses this reaction which leaves the G_α subunit incapable of GDP/GTP exchange in response to receptor stimulation. Mutation of Cys^{351} renders the G_α subunit resistant to ADP ribosylation and allows for pertussis toxin-treatment of the cells to abolish signalling through the endogenous pool of inhibitory G proteins.

Pertussis toxin-resistant fusion proteins between GPCRs and inhibitory G protein α subunits were first used by (Wise *et al.*, 1997a) and have subsequently become an important tool in the characterisation of ligand binding and G protein activation (Carr *et al.*, 1998; Wise *et al.*, 1997; Wise *et al.*, 1999; Kellett *et al.*, 1999; Dupuis *et al.*, 1999). The 1:1 stoichiometry that is a consequence of pertussis toxin abolition of endogenous G protein signalling allows for exact pharmacological calculation of the potency of ligands at the fused GPCR either in GTPase assays or in second messenger signalling assays such as the intact cell adenylyl cyclase assay discussed in chapter 3. This is possible by using radioligand saturation binding to measure the expression level of the receptor that therefore mirrors the expression level of the fused G protein.

The 5-HT_{1A} receptor has previously been fused to both $G_{i\alpha}$ and $G_{o\alpha}$ proteins with some interesting results (Kellett *et al.*, 1999; Dupuis *et al.*, 1999). It was found that in fusions between the 5-HT_{1A} receptor and $G_{i1\alpha}$ proteins, when Cys^{351} was mutated to Ile^{351}, an increase in basal GTPase activity was measured. This intrinsic constitutive activity could be suppressed by the inverse agonist spiperone by about 50%. This inverse agonist had no effect on the same fusion with the Gly^{351} mutation. It was also found that 5-HT produced an increase in V_{max} but no change in K_m for GTP in a high affinity GTPase assay using a range of GTP concentrations.

The effects of a range of 5-HT concentrations (1nM-100μM) on the seven different stable cells lines were examined in terms of high affinity GTPase activity

(Figures 5.4 and 5.5). For the 5-HT_{1A} receptor alone and the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins, in the absence of pertussis toxin, 5-HT produced a concentration dependent increase in high affinity GTPase activity (Figure 5.4). The pEC_{50} values for the 5-HT_{1A} receptor and the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion protein were not significantly different from each other ($P > 0.05$) (Table 5.1). Prior pertussis toxin treatment of membranes resulted in an abolition of agonist mediated effects with the exception of the WT $G_{i1\alpha}$ fusion protein where concentrations above 1μM produced an increase in GTPase activity. This effect was also recorded by Kellett *et al.* (1999) and may be due to insufficient pertussis toxin treatment. It may also be a reflection of the ability of high concentrations of 5-HT to overcome the inhibition of receptor-G protein coupling caused by ADP ribosylation of the G protein due to the intrinsic constitutive activity of the fusion protein.

Comparison of the 5-HT_{1A} receptor-WT G protein fusion pEC_{50} values with the non pertussis toxin-treated Gly^{351} and Ile^{351} mutant G protein fusions demonstrated that for both the $G_{i1\alpha}$ and $G_{o1\alpha}$ proteins the pEC_{50} values for the Gly^{351} mutants were significatly lower and for the Ile^{351} higher than their respective WT G protein fusions (Tables 5.1 and 5.2). These results support the findings of Bahia *et al.*, (1998) that suggested that the more hydrophobic the amino acid at position 351 of the $G_{i1\alpha}$ protein, the stronger the interaction between the receptor and G protein.

Concentration dependent increases in high affinity GTPase activity were also recorded for the pertussis toxin resistant fusion proteins (Figure 5.5). For each of the four fusion proteins, with the exception of the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ protein, pertussis toxin treatment of membranes did not result in a significant difference in pEC_{50} value when compared to non-pertussis toxin-treated samples (Tables 5.1 and 5.2). The pEC_{50} values of 5-HT stimulated high affinity GTPase activity for the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ non and pertussis toxin-treated (6.11+/-0.07nM and 5.94+/-0.08nM respectively) proteins were significantly different from those of the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein (7.23+/-0.09nM and 6.50+/-0.07nM respectively) ($P < 0.001$ and 0.01 respectively). Comparison of the other EC_{50} values did not indicate any significant differences in ability to stimulate GTPase activity, a result that could indicate that the affinity of 5-HT for the 5-HT_{1A} receptor is not significantly altered by the presence of the different G protein fusions and that the various G protein mutations may have

altered the level of intrinsic constitutive GTPase activity, but have not altered the maximum level of agonist stimulated activity.

The intrinsic GTPase activity of G protein α subunits can be altered by a family of proteins know as RGS proteins (Regulators of G protein Signalling) (Hong *et al.*, 1993). These act as GAPs (GTPase Activating Proteins) for the inhibitory class of G proteins, increasing the rate of GTP hydrolysis to GDP and thus the rate of G protein inactivation. These proteins vary widely in structure and have secondary functions, but all contain a common 120 amino acid RGS domain. It is this domain that interacts with the G_{α} subunit and increases the GTPase activity in the protein. Deletion of small sections of the RGS domains of RGS4, RGS10 and RGS GAIP was found to abolish *in vitro* GAP activity (Faurobert and Hurley, 1997; Popov *et al.*, 1997; Srinivasa *et al.*, 1998).

A number of RGS proteins have been fused to GST and transformed into bacterial BL21 cells. Following IPTG induction, the proteins are produced in large quantities and can be purified using Glutathione Sepharose 4B gel which selectively binds to the fused GST allowing separation from other bacterial proteins up to a final concentration of approximately 10-20μM.

Using fusions between the 5-HT_{1A} receptor and both $G_{i1\alpha}$ and $G_{o1\alpha}$ WT and pertussis toxin-resistant proteins the interaction between the receptor and its cognate G proteins was further characterised and the effects of two RGS proteins, RGS1 and RGS16, on these interactions examined. In fusions between the 5-HT_{1A} receptor and pertussis toxin resistant $G_{i1\alpha}$ proteins, both 5-HT and 8-OH-DPAT produced significant increases in high affinity GTPase activity ($P < 0.05$) that were not statistically different from each other (Figures 5.1-5.3) ($P > 0.05$). This effect was shown to be sensitive to the presence of RGS1 and RGS16 (1μM), both of which caused significant increases in GTPase activity at the two constructs following stimulation by either 5-HT or 8-OH-DPAT (Figure 5.4). As a control, it was assessed if RGS protein could produce any effect either in the presence or absence of 5-HT in mock transfected HEK293 cells (Figure 5.8). It was found that neither 5-HT (100μM) nor RGS1 (1μM) alone or combined could produce any change in the basal GTPase activity. To investigate this RGS protein effect further, increasing concentrations of GTP were used to quantitate V_{max} and K_m for GTP. These assays

were carried out in the absence and presence of RGS protein at basal and following 5-HT (100μM) stimulation of GTPase activity.

In membranes expressing the 5-HT_{1A} receptor alone, 5-HT produced an increase in V_{max} without effecting the K_m for GTP (Figure 5.8). RGS1 alone had no effect on basal activity, but in the presence of 5-HT produced a significant increase over 5-HT alone in both V_{max} and K_m for GTP ($P < 0.05$). The increase in K_m is not as a result of changes in the affinity of the G_α protein for GTP but due to the increased rate of GTP hydrolysis to GDP. Following pertussis toxin treatment of membranes, neither 5-HT nor RGS1 alone or together produced any significant effects on GTPase activity. These effects demonstrate that in the absence of stimulation, RGS1 protein does not effect basal GTPase activity at the 5-HT_{1A} receptor, but in the presence of 5-HT stimulation, it produces a large increase in V_{max} and also an increase in the K_m which reflects the increase in GTPase activity without any actual change in the affinity that the G protein has for GTP. This lack of effect of RGS proteins in the 5-HT_{1A} receptor means that there is not a significant degree of constitutive activity in these membranes.

When the WT $G_{i1\alpha}$ and $G_{o1\alpha}$ proteins are fused to the 5-HT_{1A} receptor and stably expressed in HEK293 cells, the effects seen are slightly different to the receptor alone (Figure 5.9 A + B). There is an increase in basal GTPase activity that reflects an increase in intrinsic GTPase activity brought on by the close proximity of the G protein to the receptor. 5-HT alone produces a significant increase in high affinity GTPase activity over basal ($P < 0.05$), an increase that is equalled by the presence of RGS1 alone ($P < 0.05$). RGS protein can cause an increase in GTPase activity in the absence of agonist stimulation the 5-HT_{1A} receptor WT G protein fusions. This increase in GTPase activity is a reflection of the constitutive activity caused by increased likelyhood of agonist independent interaction of the receptor and G protein due to their close proximity . RGS protein alone also produces a significant increase in the K_m for GTP which reflects the increase in hydrolysis of GTP to GDP and not an increase in the rate of exchange of GDP for GTP. There is a more dramatic increase in GTPase activity in the presence of RGS1 following stimulation with 5-HT, producing significant increases over stimulation with either 5-HT or RGS1 protein alone ($P < 0.05$). In the presence of pertussis toxin, the basal V_{max} for both WT G protein fusions was significantly decreased over untreated basal activity.

This reflects the inability of the receptor to stimulate GDP/GTP exchange following ADP ribosylation at Cys^{351} of the G protein. Stimulation by 5-HT, RGS1 and the combination of the two is still seen, a reflection of the high affinity of the fused G protein for the $5\text{-}HT_{1A}$ receptor, but these have been significantly reduced below untreated membranes ($P < 0.05$).

The results from these data give an indication of GTPase activity in the presence and absence of RGS proteins, but as it is not possible to show only coupling to the fused G protein, the value of the pertussis toxin-resistant G protein fusions becomes apparent. For the following experiments using the $5\text{-}HT_{1A}/G_{i1\alpha}C^{351}G/I$ and $5\text{-}HT_{1A}/G_{o1\alpha}C^{351}G/I$ fusion proteins all cells were treated prior to harvest with pertussis toxin to prevent any signalling through the endogenous pool of inhibitory G proteins.

A wide range of RGS protein concentrations were examined, from 1nM to 5μM for RGS1 and from 1nM to 1μM for RGS16. Their effects were similar on the two Gly^{351} mutants and on the two Ile^{351} mutants. For both RGS proteins, incubation with membranes expressing the $5\text{-}HT_{1A}/G_{i1\alpha}C^{351}G$ and $5\text{-}HT_{1A}/G_{o1\alpha}C^{351}G$ in the absence of agonist stimulation had no effect on either V_{max} or the K_m for GTP with the exception of 1μM RGS1 which significantly increased the V_{max} for the $G_{o1\alpha}$ Gly^{351} mutant without any effect on K_m (Tables 5.3 and 5.5). This reflects the low intrinsic constitutive GTPase activity of these two constructs and supports the findings of Kellett *et al.* (1999) where the inverse agonist spiperone had no effect on basal GTPase activity in membranes expressing the $5\text{-}HT_{1A}/G_{i1\alpha}C^{351}G$ fusion protein.

In the presence of 100μM 5-HT alone, high affinity GTPase activity is significantly greater than basal activity for both of the Gly^{351} mutant fusion proteins ($P < 0.05$). Increasing levels of both RGS1 and RGS16 result in a concentration dependent increase in V_{max} for both the $G_{i1\alpha}$ and $G_{o1\alpha}$ mutant fusions with the effects becoming significant at 50nM for RGS1 and 5nM for RGS16 (Tables 5.3 and 5.5). K_m increases in response to rising concentrations of RGS protein but these effects only become significant for the $G_{i1\alpha}$ mutant at 1μM for RGS1 and 100nM for RGS16. Effects on the K_m for GTP for the $G_{o1\alpha}$ mutant fusion become significant at 50nM for both RGS1 and RGS16.

In membranes expressing the $5\text{-}HT_{1A}/G_{i1\alpha}C^{351}G$ fusion protein, for both RGS1 and RGS16 GTP does not reach saturating concentrations at the highest

concentrations of RGS protein, reflecting the ability of the higher RGS protein concentrations to increase the rate of GTP hydrolysis (Figure 5.18, 5.20, 5.24, 5.28-30). In membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ fusion protein, this effect is more pronounced, possibly indicating that the $G_{o1\alpha}$ protein is capable of hydrolysing GTP at a greater maximal rate that the $G_{i1\alpha}$ protein or that the RGS proteins have a higher affinity for $G_{o1\alpha}$ that $G_{i1\alpha}$ protein. This may be supported by the general finding in the literature that the 5-HT_{1A} receptor has a greater ability to signal through $G_{i\alpha}$ proteins that $G_{o\alpha}$ proteins (Clawges *et al.*, 1997; Bertin *et al.*, 1992; Garnovskaya *et al.*, 1997; Raymond *et al.*, 1993). A possible explanation is that the greater rate of GTP hydrolysis at the $G_{o\alpha}$ protein terminates signalling of second messenger systems before termination by the $G_{i\alpha}$ protein that with a lower rate of GTP hydrolysis remains activated for a longer period.

When expression levels of the two receptor-G protein constructs are taken into account, neither RGS protein had a significant effect on basal turnover of GTP (molecules of GTP/G protein/minute) (Figure 5.28 and 5.29). Following the addition of 5-HT, RGS1 and RGS16 had similar potency at the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ and the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ receptor G fusion proteins. In Figures 5.30 and 5.31 the basal turnover of GTP for each construct at each RGS protein concentration is subtracted from that of the 5-HT stimulated turnover of GTP to allow accurate quantitation of the effects of RGS protein on 5-HT stimulated high affinity GTPase activity. From Figure 5.30 it appears that RGS1 and RGS16 have identical effects on 5-HT stimulated GTPase activity, neither having reached a concentration of RGS protein that produces a maximal effect. The predictions of a maximum for the effects of RGS protein are different however, with RGS1 having a predicted 32 fold maximal effect on $G_{i1\alpha}C^{351}G$ GTPase activity and RGS16 only a 4.4 fold effect.

The results for the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ construct seemed to show that RGS1 stimulates increases in GTPase activity at lower concentrations, but this effect was not however statistically significant over the effects of RGS16. The increases in 5-HT stimulated GTPase activity seemed to have reached a plateau for RGS1 but not for RGS16, with RGS1 having a predicted maximal 12.4 fold stimulation of GTP turnover and RGS16 a 189 fold maximum. These figures may not be accurate, considering that the curves they are predicted from are only reaching the exponential phase in some cases. It may be realistic to assume, that as the rises in GTPase

activity stimulated by the presence of RGS1 and RGS16 have not been statistically different from one another, that they will both show a similar maximal effect on GTP hydrolysis.

The effects of the Ile^{351} G protein – 5-HT_{1A} receptor fusions were quite different. As with the Gly^{351} mutants, the effects of RGS1 and RGS16 over a range of concentrations on both basal and 5-HT stimulated GTPase activity were examined over a range of GTP concentrations. Unlike the Gly^{351} mutants, the basal GTPase activity of the Ile^{351} mutants was affected by the addition of increasing concentrations of both RGS1 and RGS16. For the $G_{i1\alpha}$ fusion protein, this was reflected by a significant increase in V_{max} at only 5nM for RGS1 and a twofold, but not statistically significant increase in V_{max} for RGS16 (Tables 5.4 and 5.6). Both RGS1 and RGS16 produced statistically significant increases in V_{max} for the Go1α fusion protein at 10nM in the absence of 5-HT stimulation. Although increases in the K_m for GTP for both the $G_{i1\alpha}$ and the $G_{o1\alpha}$ fusion proteins were measured, only RGS1 produced a statistically significant increase in K_m for the $G_{o1\alpha}$ fusion protein at concentrations of above 100nM. The curves generated in the presence of increasing concentrations of GTP did not saturate at approximately 3000nM in the presence of the highest concentrations of both RGS1 and RGS16 when incubated with the $G_{i1\alpha}$ and the $G_{o1\alpha}$ Ile^{351} fusion proteins. This might indicate that these levels of RGS protein are capable, in the presence of the membranes expressing the G protein fusion, of increasing the hydrolysis of a greater concentration of GTP to GDP than was present in the assay.

In the presence of 5-HT (100μM) the RGS proteins have an even more dramatic effect on high affinity GTPase activity at both the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ and 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion proteins. The increase in GTPase activity stimulated by the presence of 5-HT alone was significantly greater than basal for the two fusion proteins ($P < 0.05$) with out affecting the K_m for GTP. For the $G_{i1\alpha}$ Ile^{351} mutant, the presence of RGS1 produced a significant increase in V_{max} and K_m at 100nM ($P = 0.0083$ and 0.0303 respectively) (Tables 5.4 and 5.6). In the presence of RGS16, there was a significant increase in V_{max} at 1nM and in K_m at 5nM. This does not appear to reflect a difference in affinity of the RGS proteins for the $G_{i1\alpha}$ protein, as neither produce statistically different effects on the turnover of GTP once expression

levels are taken into account either at basal levels or following 5-HT stimulation (Figures 5.28).

For the $G_{o1\alpha}$ Ile^{351} protein, in the presence of 5-HT, increasing concentrations of RGS1 produced a significant elevation of V_{max} and K_m at 1nM and 50nM respectively ($P < 0.05$) and in the presence of RGS16 at 5nM for both V_{max} and K_m. As with the $G_{i1\alpha}$ Ile^{351} fusion protein the effects of neither RGS protein on turnover of GTP to GDP once expression level were accounted for produced statistically different effects from one another in the absence or presence of 5-HT stimulation (Figure 5.29). Once the effects on basal GTPase activity have been subtracted from those in the presence of 5-HT, RGS1 produces a predicted 64-fold increase in turnover number over 5-HT stimulation for the $G_{o1\alpha}$ construct and RGS16 a 1812 fold increase (Figures 5.30 and 5.31). For the $G_{o1\alpha}$ fusion, RGS1 has a predicted fold stimulation of 209 and RGS16 a predicted 19-fold increase over 5-HT stimulated turnover. As with the predictions for the Gly^{351} mutants these figures may not be accurate as the concentration response curves to RGS1 and RGS16 have not saturated and are for the most case still in the exponential phase of effect. Again, it would possibly be more accurate to predict that due to the lack of statistically significant differences between the effects of RGS1 and RGS16 at the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins, their affinity for both G proteins appears to be similar, and they will most likely have similar maximal effects on GTP turnover.

The effects of RGS1 and RGS16 proteins on high affinity GTPase activity were different for the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins. Both RGS proteins produced greater increases in stimulated turnover for both the Gly^{351} and Ile^{351} $G_{o1\alpha}$ fusion proteins than for the Gly^{351} and Ile^{351} $G_{i1\alpha}$ fusion proteins (Figures 5.31 and 5.32). The effects of maximal concentrations of RGS protein on the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ protein were significantly greater than the effects on the 5-$HT_{1A}/G_{i1\alpha}C^{351}G$ fusion protein ($P < 0.05$). The increase in GTP turnover for the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ protein in the presence of 5μM RGS1 was significantly greater than for the $G_{i1\alpha}C^{351}I$ protein ($P < 0.05$). The effects of RGS16, which appeared to produce a larger stimulation of GTP turnover for the $G_{o1\alpha}C^{351}I$ fusion protein compared to the $G_{i1\alpha}C^{351}I$ fusion, were not significantly different, a result that reflects the large error in the turnover values

($P > 0.05$). These results may be a reflection of RGS1 and RGS16 having a greater affinity for the 5-$HT_{1A}/G_{o1\alpha}$ fusion proteins than for the 5-$HT_{1A}/G_{i1\alpha}$ fusion proteins.

It has been clearly demonstrated that agonist activation of GTPase activity has no effect on the K_m for GTP of the stimulated G protein (Figures 5.14, 5.16, 5.18, 5.20, 5.24, 5.26, 5.28 and 5.30). However, in the presence of high concentrations of RGS protein, a significant increase in K_m is measured that is due to the increase in the rate of GTP hydrolysis, but not any change in affinity for GTP/GDP. With this clear shift in K_m in the presence of RGS protein, it could be asked why there is no shift in the absence of RGS protein, a shift that would represent the endogenous pool of RGS proteins? To investigate this lack of endogenous RGS protein effect, a number of simple experiments were carried out.

Firstly, using the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ stable cell line, the fractions that are normally discarded during the preparation of cell membranes were assayed to check if they possessed any RGS protein activity as RGS proteins are predicted to be mainly cytoplasmic proteins (Burchett 2000). However, following addition of the cytosolic fractions normally discarded to reaction mixes already containing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ membranes, a decrease in basal and stimulated GTPase activity were measured.

Secondly, it was decided to see if pre-treatment of the cells with 5-HT (100μM) could stimulate the 5-HT_{1A} receptor and recruit the RGS protein to the membrane where it might then be held more securely during membrane preparation. It has been shown previously that receptor activation results in the recruitment of RGS3, RGS4 and RGS16 to the plasma membrane from the cytoplasm (Druey *et al.*, 1998; Chen *et al.*, 1999; Dulin *et al.*, 1999). However, no statistical difference was found between the treated and non-treated membranes in a GTPase assay.

In order to investigate the endogenous expression of RGS proteins further, it was decided to make use of Taqman QrtPCR, a method that allows real-time quantitation of specific nucleic acid sequences using a flourogenic 5'nuclease assay. In this assay, the probe consists of a oligonucleotide, with a reporter and quencher dye attached, complementary to the target sequence that is added to the PCR reaction mixture. During PCR, if the target of interest is present, the probe anneals specifically between the forward and reverse primer sites. The neucleolytic activity of the polymerase cleaves the probe, resulting in increased fluorescent intensity from

the reporter dye. This occurs in every cycle of the PCR reaction and does not interfere with the accumulation of the PCR product.

Primers and probes were designed for a number of RGS proteins reported to be ubiquitously expressed, or expressed in the organ of interest, the kidney (Table 5.7). Following problems with the standard curves run using cDNA samples for the RGS proteins of interest, it was only possible to conclude that RGS2, RGS3 and RGS4 proteins are expressed in HEK293 cells and that the expression is most probably at low levels. This may give some indication as to why no endogenous RGS protein activity is detectable in the GTPase assay, i.e. expression levels of RGS proteins are so low in comparison to those of the receptor and G protein of interest that any effect they may have is no significant enough to measure using current techniques.

Both RGS1 and RGS16 proteins had a significant effect on the basal GTPase activity for both the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins. It was decided to examine the effects of a range of ligands, including both agonists and inverse agonists on high affinity GTPase activity, to see if the effects of the RGS proteins would be of benefit in the design of a robust ligand screening assay.

Chapter 6

High Affinity GTPase Assays With RGS1 Protein as a Ligand Screen

6.1 Introduction

The concept of inverse agonism in GPCRs was first explored in the late 1980's through work done on the δ opioid receptor (Costa and Herz, 1989). They demonstrated that some antagonists for the δ opioid receptor had "negative intrinsic activity" *in vitro*, contrasting with others that lacked any intrinsic activity. An inverse agonist puts a receptor into a conformation that favours the G protein-uncoupled state, an agonist, the G protein-coupled state and an antagonist does not favour coupling to either form (De Lean *et al.*, 1980).

At the human 5-HT_{1A} receptor, the ligand spiperone has been previously reported as acting as an inverse agonist (Barr and Manning, 1997; Newman-Tancredi *et al.*, 1997; Kellett *et al.*, 1999). A number of other ligands have also been reported to show inverse agonist activity at the 5-HT_{1A} receptor, including methiothepin and (+)butaclamol (Newman-Tancredi *et al.*, 1998; Stanton and Beer, 1997). The dopamine D_2 selective antagonists chlorpromazine and haloperidol bind to 5-HT receptors with lower affinity and have previously been shown to have inverse activity at 5-HT_{1B} and 5-HT_{1D} receptors (Audinot *et al.*, 2001). Together with 5-HT, spiperone, and WAY100635 (Routledge, 1996), the ability of haloperidol, chlorpromazine, butaclamol, methiothepine and another dopamine antagonist thioridazine to effect GTPase activity of the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ and 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusions will be examined.

Kellett *et al.* (1999) showed using fusion proteins between the 5-HT_{1A} receptor and $G_{i1\alpha}$ either WT or Ile^{351} protein that spiperone produced a concentration dependent decrease in basal GTPase activity of membranes expressing this construct. The mutants will be used due to their increased intrinsic basal GTPase activity. This mutation leads to an increase in hydrophobicity at this residue and an increase in affinity of the receptor for the G protein (Chapter 4). This effect alone allows for increased ability to distinguish between antagonists at the receptor and partial agonists, but coupled with the increase in basal GTPase activity previously discussed in the presence of RGS proteins, this produces a robust assay that allows for screening for agonist/inverse agonist/antagonist activity under the same conditions.

Antagonists for the 5-HT_{1A} receptor enhance the antidepressant effects of selective serotonin uptake inhibitors (SSRIs) such as fluoxetine (Prozac) (Artigas *et al.*, 1994; Artigas *et al.*, 1996). This action is thought to be due to inhibition of 5-HT

activation of presynaptic 5-HT_{1A} receptors, activation of which leads to decreased 5-HT release. It is therefore important to identify the exact characteristics of the co-administered 5-HT_{1A} ligand because agonist could result in the downregulation of receptor expression with time whereas inverse agonists may have the opposite effect.

The ability of the high affinity GTPase assay using constitutively active 5-HT_{1A} receptor-G protein fusions in the presence of RGS proteins to distinguish between different ligand types was examined in this final section of work.

6.2 Results

Increasing concentrations of the ligand spiperone have been previously shown to decrease basal GTPase activity at the 5-HT_{1A} receptor fused to either WT $G_{i1\alpha}$ protein or Ile^{351} mutant $G_{i1\alpha}$ protein (Kellett *et al.*, 1999). No effect was demonstrated at the 5-HT_{1A} receptor alone or the $G_{i1\alpha}$ Gly^{351} fusion protein. This effect was completely abolished by pertussis toxin-treatment of membranes containing the WT fusion, but the toxin had no effect on the Ile^{351} mutant fusion, indicating that the effect was due to activation of $G_{i\alpha}$ proteins and not another undescribed mechanism.

The inverse agonist properties of spiperone and a number of other ligands were further investigated in the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ and 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusions in the absence and presence of RGS1. In membranes expressing these two 5-HT_{1A} receptor-G protein constructs, RGS1 produced concentration-dependent increases in basal GTPase activity (Figure 6.1) with the effects having EC_{50} values of 90nM for the $G_{i1\alpha}$ fusion protein and 52nM for the $G_{o1\alpha}$ fusion protein. Using a maximal concentration of RGS1 protein produced an increase in basal GTPase activity providing a larger window of opportunity to assess the effects of a range of ligands. In parallel, competition binding curves were performed with the radioligand [^{3}H]-WAY100635, allowing the pK_i for each ligand to be calculated.

The effects of a range of concentrations of 5-HT (1nM – 100μM) were examined initially on the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein (Figure 6.2, A). There was no significant difference in the EC_{50} for 5-HT activation of GTPase activity in the absence or presence of RGS1 (41nM +/- 4.5 and 132 +/- 20.8nM respectively) (Table 6.1), but from producing less than a two fold increase in GTPase activity over basal in the absence of RGS1, 5-HT produced a greater than five fold increase in GTPase activity over basal in the presence of RGS1. RGS1 on its own produced less than a two fold increase in basal GTPase activity in membranes expressing the $G_{i1\alpha}$ fusion protein but in membranes expressing the $G_{o1\alpha}$ fusion protein, this elevation of basal GTPase activity was nearly four fold (Figure 6.3, A). The effects of 5-HT on the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion protein were similar; less than a two fold stimulation of GTPase activity over basal in the absence of RGS1, but a greater than ten fold increase in GTPase activity over basal in the presence of RGS1. The estimated pK_i

for 5-HT was 7.12 for the $G_{i1\alpha}$ fusion and 7.00 for the $G_{o1\alpha}$ fusion, figures consistent with those found in the literature (Newman-Tancredi *et al.*, 2001) (Table 6.2).

Previously, WAY100635 has been reported as a neutral antagonist at the 5-HT_{1A} receptor (Fletcher *et al.*, 1996; Newman-Tancredi *et al.*, 1997; Routledge 1996), but in these studies using the 5-HT_{1A} receptor-G protein fusions with constitutive activity it acted as a partial agonist (Figures 6.4, and 6.5 A). At both the $G_{i1\alpha}$ and $G_{o1\alpha}$ Ile^{351} fusions in the absence of RGS1, it showed very weak partial agonist activity, elevating the basal GTPase activity slightly (less than 1.5 fold), but in the presence of RGS1 this effect was exaggerated and its partial agonist activity became more pronounced, particularly with the $G_{o1\alpha}$ fusion (Figure 6.5 A). Importantly, RGS1 had no effect on the binding of [^{3}H]-WAY100635 to the constructs, either in terms of K_d or B_{max} (Figure 6.6 A + B). The estimated pK_i for WAY100635 at both the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins (8.92 and 9.05 respectively) was consistent with those reported in the literature (Newman-Tancredi *et al.*, 2001).

Previously published data on the 5-HT_{1A}/$G_{i1\alpha}$ fusion proteins demonstrated that spiperone had inverse agonist activity at the 5-HT_{1A}/$G_{i1\alpha}$ and 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion proteins (Kellett *et al.*, 1999), resulting in approximately 50% reduction in basal GTPase activity. Both of these fusions show basal constitutive activity, with the Ile^{351} fusion showing the greatest.

Increasing concentrations of spiperone (1nM – 100μM) were examined for effects on basal GTPase activity in membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein or the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion protein (Figures 6.7 and 6.8). In the absence of RGS1, spiperone produced a clear reduction in basal GTPase activity for the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion, but this effect was less pronounced for the $G_{o1\alpha}$ Ile^{351} fusion protein (approximate 50% and 30% reductions respectively). The effects of RGS1 are not to increase the rate of exchange of GDP for GTP on the G_α subunit, only to increase the rate of GTP hydrolysis. Given this, it was unsurprising that the effects of spiperone became exaggerated in the presence of RGS protein as the basal rate of GTPase activity was increased over 1.5 fold for 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ and four-fold for 5-HT_{1A}/$G_{o1\alpha}C^{351}I$. It was now possible to measure accurately the potency of spiperone at these fusion proteins, with EC_{50} values of 46 +/- 6.8nM and 32 +/- 13nM respectively in the presence of RGS1 (Table 6.1). The pK_i values for spiperone were consistent with those found in the literature (Table 6.2) (Newman-Tancredi *et*

al., 2001). It was also noted that the affinity of spiperone for the fusion proteins was unaffected by the presence of RGS1 (Figure 6.9) with no change in the IC_{50} values for displacement of [^{3}H]-WAY100635 binding.

To further explore the effects of the RGS protein on basal GTPase activity and the effects of spiperone on these, high affinity GTPase assays were carried out over a wide range of GTP concentrations (Figures 6.10 and 6.11) (Table 6.3). Basal GTPase activity was elevated in the presence of RGS1 alone, again 1.5 fold in the case of the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion and 4 fold for the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion in terms of V_{max}. Spiperone produced a significant decrease in basal V_{max} in the absence of RGS1 for both $G_{i1\alpha}$ and $G_{o1\alpha}$ constructs (P = 0.0308 and 0.0006 respectively) and a significant decrease in RGS1 increased basal V_{max} for both (0.0075 and 0.0005 respectively). As a control, 5-HT also increased V_{max} over basal and significantly increased V_{max} over RGS1 increased basal GTPase activity for both G_i and G_o fusion proteins ($P < 0.05$).

The effects of a number of other ligands on basal GTPase activity in membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ or $G_{o1\alpha}C^{351}I$ fusions were examined. Methiothepine (Figures 6.12 and 6.13), chlorpromazine (Figures 6.14 and 6.15), (+)-butaclamol (Figures 6.16 and 6.17), and thioridazine (Figures 6.18 and 6.19) all demonstrated inverse agonist activity on basal GTPase levels. These effects were clearly visible in membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein, but as with spiperone it was not possible to quantitate the effects accurately. In the presence of RGS1 though, the 1.5 fold increase in basal GTPase activity for the $G_{i1\alpha}$ fusion and the 4 fold increase for the $G_{o1\alpha}$ fusion, made it possible to measure the potencies of these inverse agonists at these constructs. These results are summarised in table 6.1 with pEC_{50} and pK_i values summarised in table 6.2.

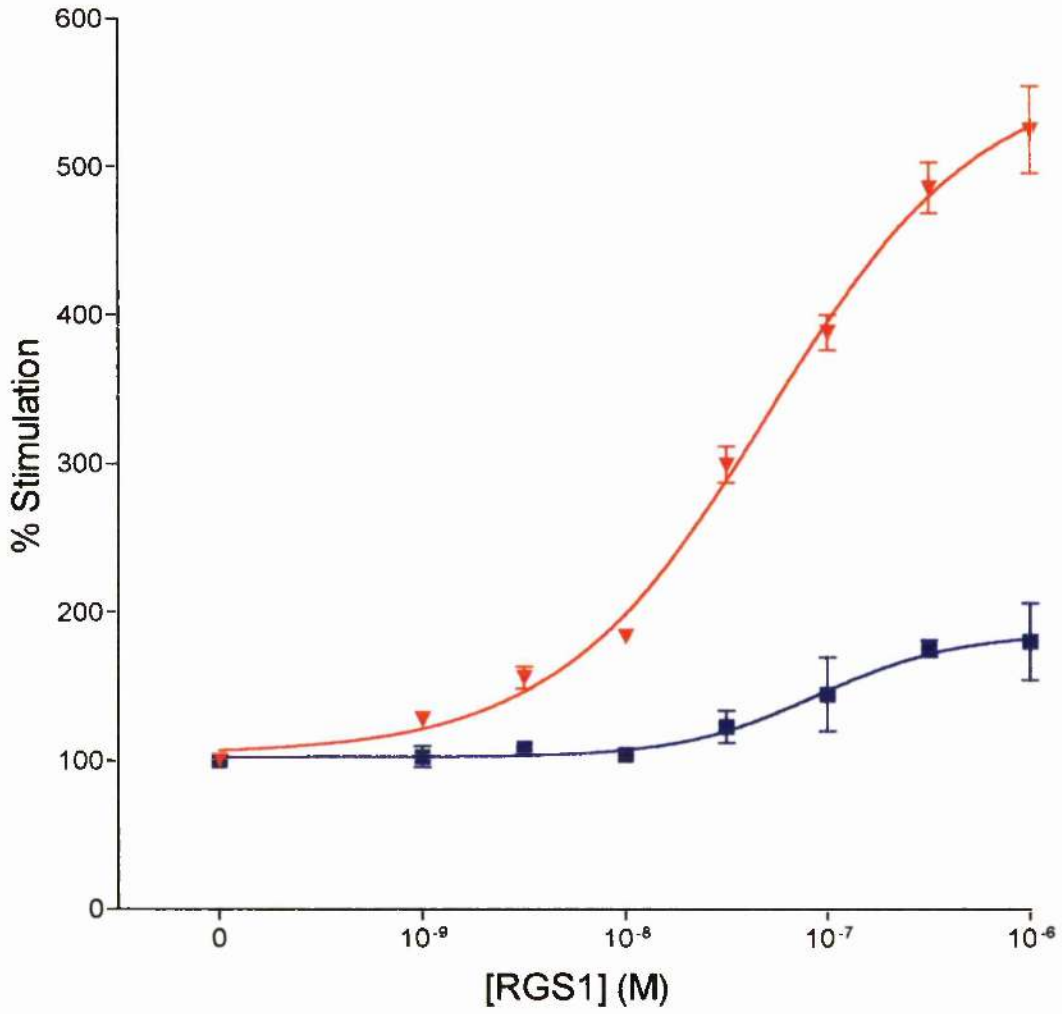
Only in the case of haloperidol was a lack of significant inverse agonist activity found at both the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins (Figure 6.20 and 6.21). This was also noted in the presence of RGS1 indicating that this ligand has zero activity at the 5-HT_{1A} receptor in these assay conditions. This was not due to a lack of receptor binding, illustrated by the ability of haloperidol to compete with [^{3}H]-WAY100635 for binding to the 5-HT_{1A} receptor (Figures 6.20B and 6.21B). Haloperidol acted as an antagonist at the 5-HT_{1A} receptor. The calculated pK_i figure was similar to those found in the literature (Newman-Tancredi *et al.*, 2001; Millan 2000) (Table 6.2).

Figure 6.1 **The effects of increasing concentrations of RGS1 protein on high affinity GTPase activity of membranes stably expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins**

RGS1 protein produced a concentration-dependent increase in high affinity GTPase activity in membranes expressing either the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ or 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins. Data are means of triplicate determinations, are representative of at least three similar experiments and are expressed as % increase over basal GTPase activity.

■ 5-$HT_{1A}/G_{i1\alpha}C^{351}I$

▽ 5-$HT_{1A}/G_{o1\alpha}C^{351}I$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200
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10-6
[RGS1] (M)

Figure 6.2 **Panel A: Effects of increasing concentrations of 5-HT on the high affinity GTPase activity of membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by 5-HT for [^{3}H]-WAY100635 binding to the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein.**

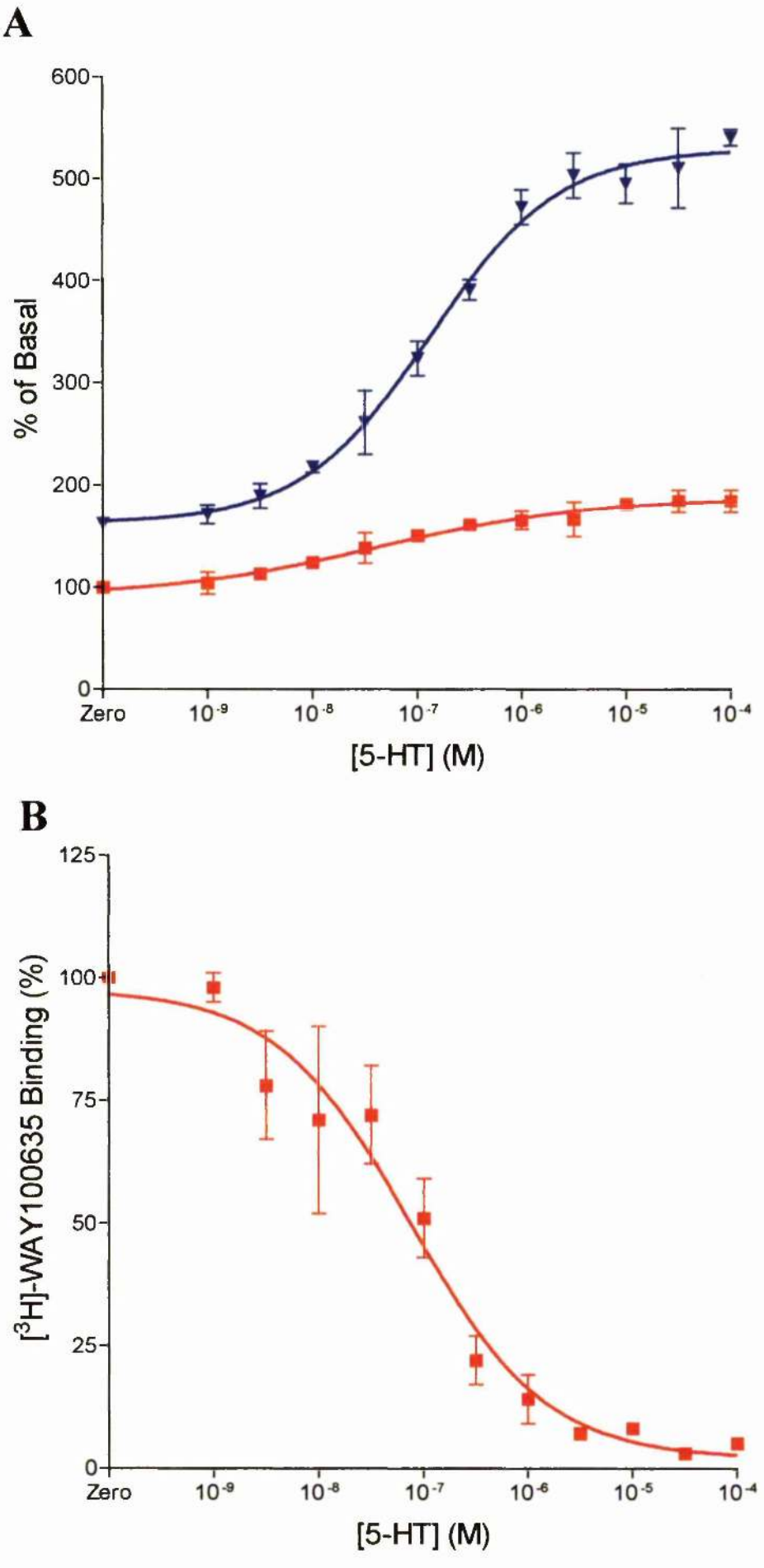
Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of 5-HT (1nM – 100μM) in the absence and presence of RGS1 (1μM).

■ 5-HT

▼ 5-HT + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of 5-HT expressed as a percentage of radioligand binding in the absence of 5-HT.

Data are means of triplicate determinations and are representative of at least three similar experiments.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[5-HT] (M)
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[3H]-WAY100635 Binding (%)
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10-6
10-5
10-4
[5-HT] (M)

Figure 6.3 **Panel A: Effects of increasing concentrations of 5-HT on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by 5-HT for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.**

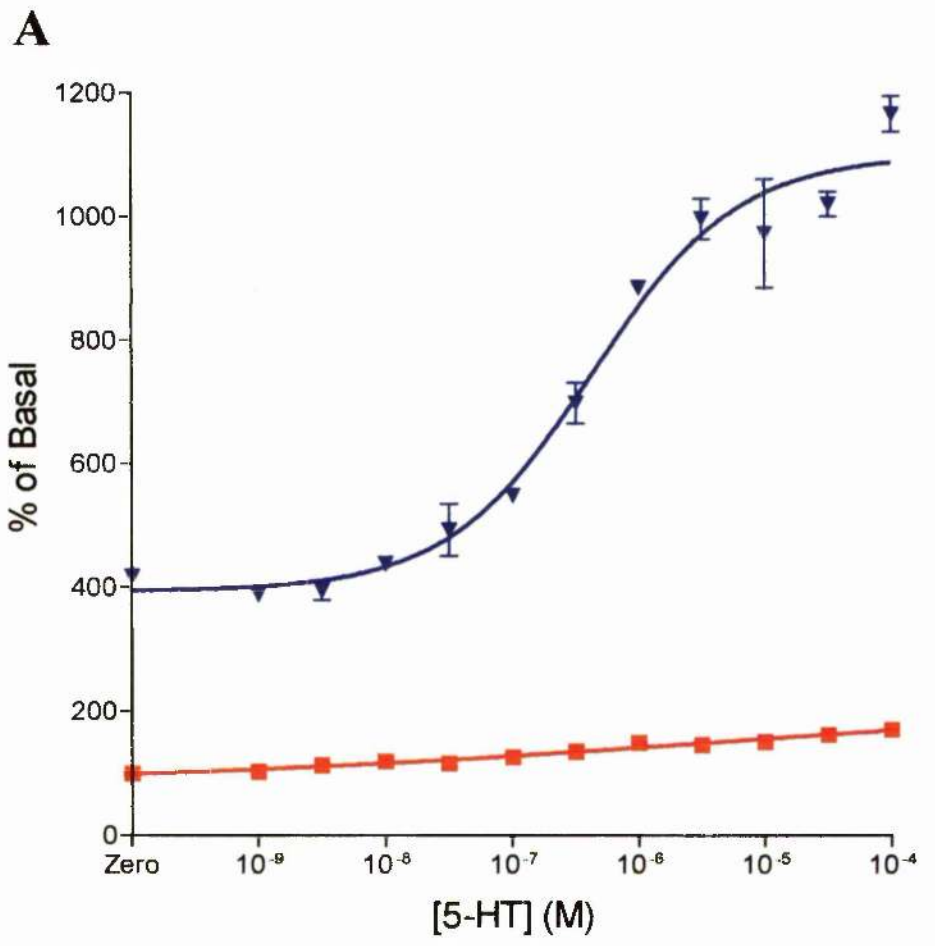
Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of 5-HT (1nM – 100μM) in the absence and presence of RGS1 (1μM).

■ 5-HT

▼ 5-HT + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of 5-HT expressed as a percentage of radioligand binding in the absence of 5-HT.

Data are means of triplicate determinations and are representative of at least three similar experiments.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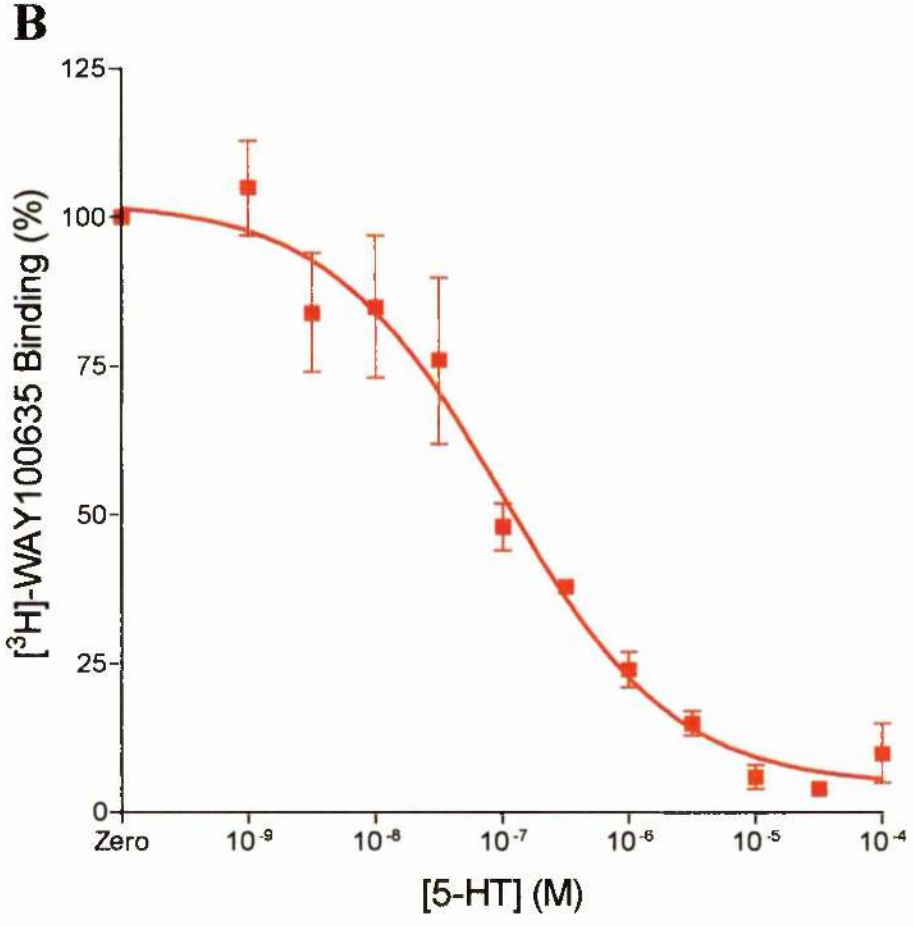
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10-6
10-5
10-4
[5-HT] (M)

Table 6.1 **Summary of EC_{50} values for ligand inhibition of basal GTPase activity in the presence and absence of RGS1 for the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ and 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion proteins**

The effects of increasing concentrations of 5-HT, WAY100635, spiperone, methiothepine, chlorpromazine, butaclamol, thioridazine and haloperidol on basal and RGS1 (1μM) stimulated GTPase activity in pertussis toxin treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ and 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion proteins were measured in terms of EC_{50}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

Ligand	5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I EC$_{50}$	5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I EC$_{50}$ (+RGS1)	5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I EC$_{50}$	5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I EC$_{50}$ (+RGS1)
5-HT	41+/-4.5	132+/-20.8	158+/-13	429+/-37
WAY100635	8+/-0.9	5+/-0.8	1+/-0.1	3+/-0.4
Spiperone	67+/-4.3	46+/-6.8	285+/-42	32+/-13
Methiothepine	21+/-1.4	30+/-2.3	33+/-0.9	24+/-1.3
Chlorpromazine	825+/-53	1433+/-85	*	865+/-48
Butaclamol	85+/-2.4	84+/-5.1	36+/-4.1	84+/-12
Thioridazine	293+/-35	97+/-28	*	213+/-11
Haloperidol	*	*	*	*

* Denotes unreliable curve fit.
All values are nM

Table 6.2 **Summary of pEC_{50} values for ligand inhibition of basal GTPase activity in the presence and absence of RGS1 for the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins. pK_i values are also show from ligand displacement of [^{3}H]-WAY100635 binding**

The effects of increasing concentrations of 5-HT, WAY100635, spiperone, methiothepine, chlorpromazine, butaclamol, thioridazine and haloperidol on basal and RGS1 (1μM) stimulated GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins were measured in terms of pEC_{50}. Ligand displacement of [^{3}H]-WAY100635 binding to the 5-HT_{1A} receptor–G protein fusions allowed calculation of pK_i figures.

Data are means of triplicate determinations and are representative of at least three similar experiments.

Ligand	5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I			5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I		
	pEC$_{50}$	pEC$_{50}$ (+RGS1)	pK_i	pEC$_{50}$	pEC$_{50}$ (+RGS1)	pK_i
5-HT	7.39	6.88	7.12	6.80	6.37	7.00
WAY100635	8.10	8.30	8.92	9.00	8.52	9.05
Spiperone	7.17	7.34	6.41	6.55	7.50	6.77
Methiothepine	7.68	7.52	7.77	7.48	7.62	8.70
Chlorpromazine	6.08	5.84	5.88	*	6.06	5.98
Butaclamol	7.07	7.08	7.11	7.44	7.08	6.24
Thioridazine	6.53	7.01	7.04	*	6.67	6.55
Haloperidol	*	*	6.49	*	*	6.33

* Denotes unreliable curve fit.

Figure 6.4 **Panel A: Effects of increasing concentrations of WAY100635 on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by WAY100635 for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of WAY100635 (0.1nM – 10μM) in the absence and presence of RGS1 (1μM).


WAY100635


WAY100635 + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of WAY100635 expressed as a percentage of radioligand binding in the absence of WAY100635.

Data are means of triplicate determinations and are representative of at least three similar experiments.

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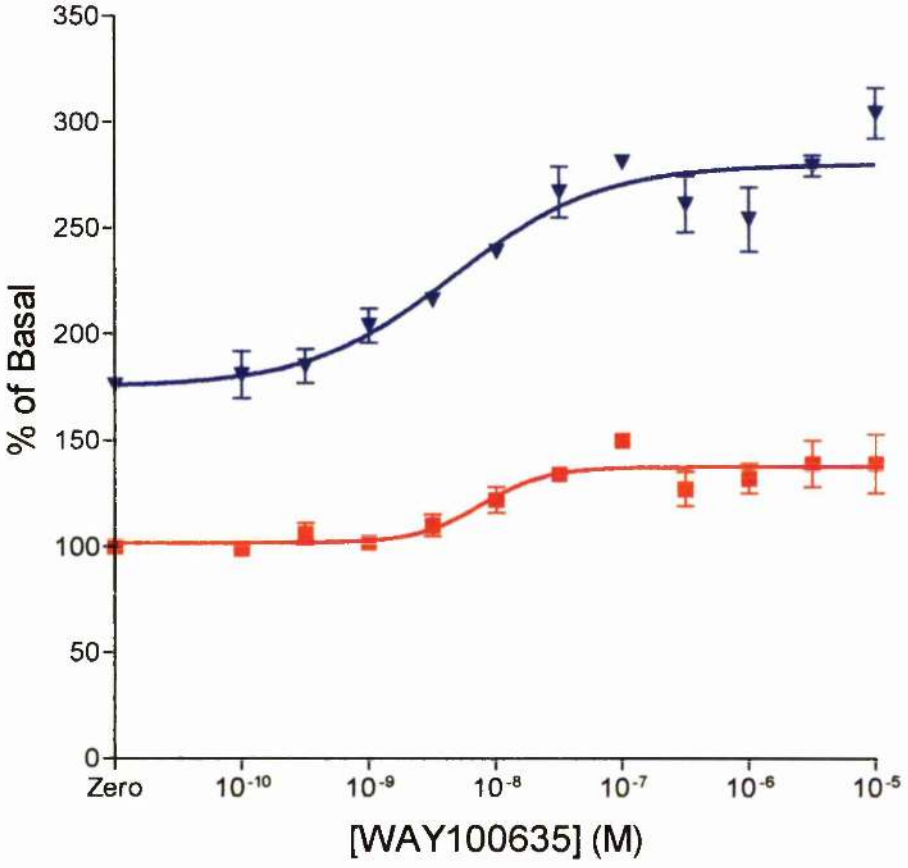
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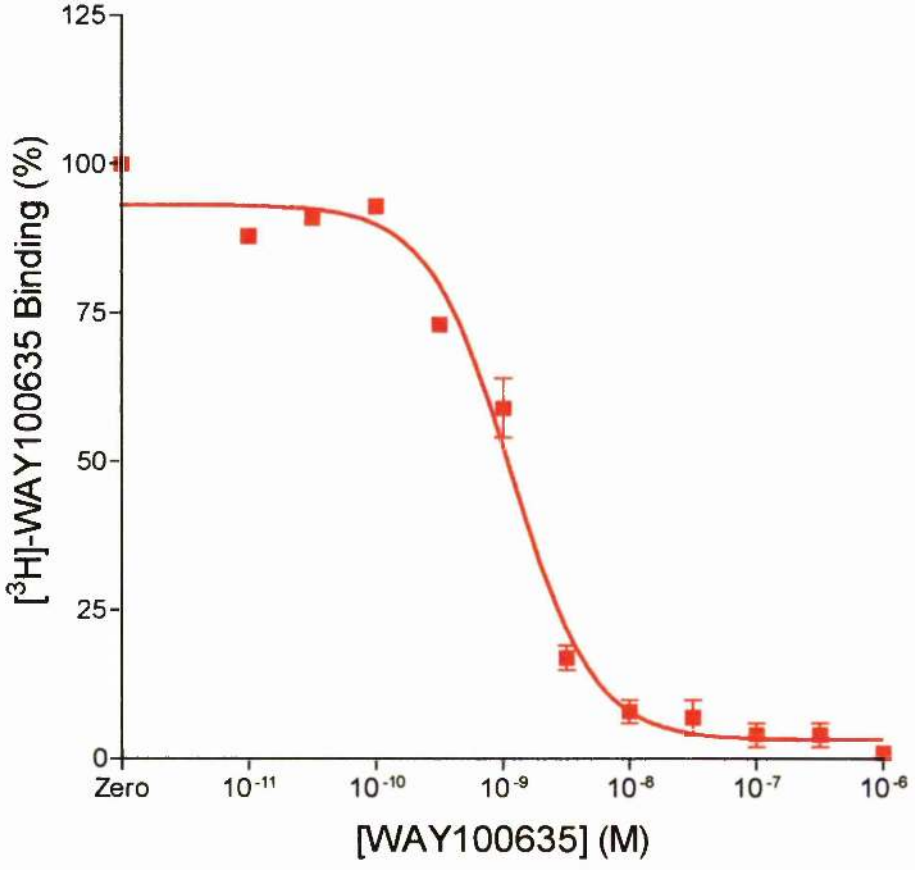
B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[WAY100635] (M)

Figure 6.5 **Panel A: Effects of increasing concentrations of WAY100635 on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by WAY100635 for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of WAY100635 (0.1nM – 10μM) in the absence and presence of RGS1 (1μM).

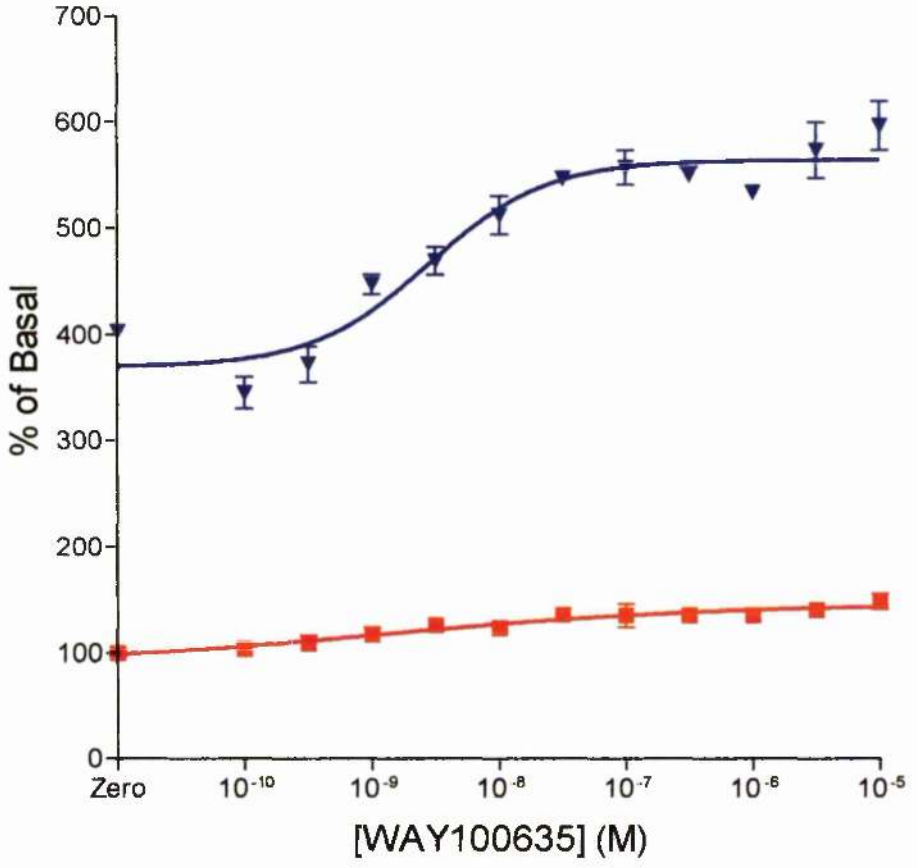
WAY100635

WAY100635 + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of WAY100635 expressed as a percentage of radioligand binding in the absence of WAY100635.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A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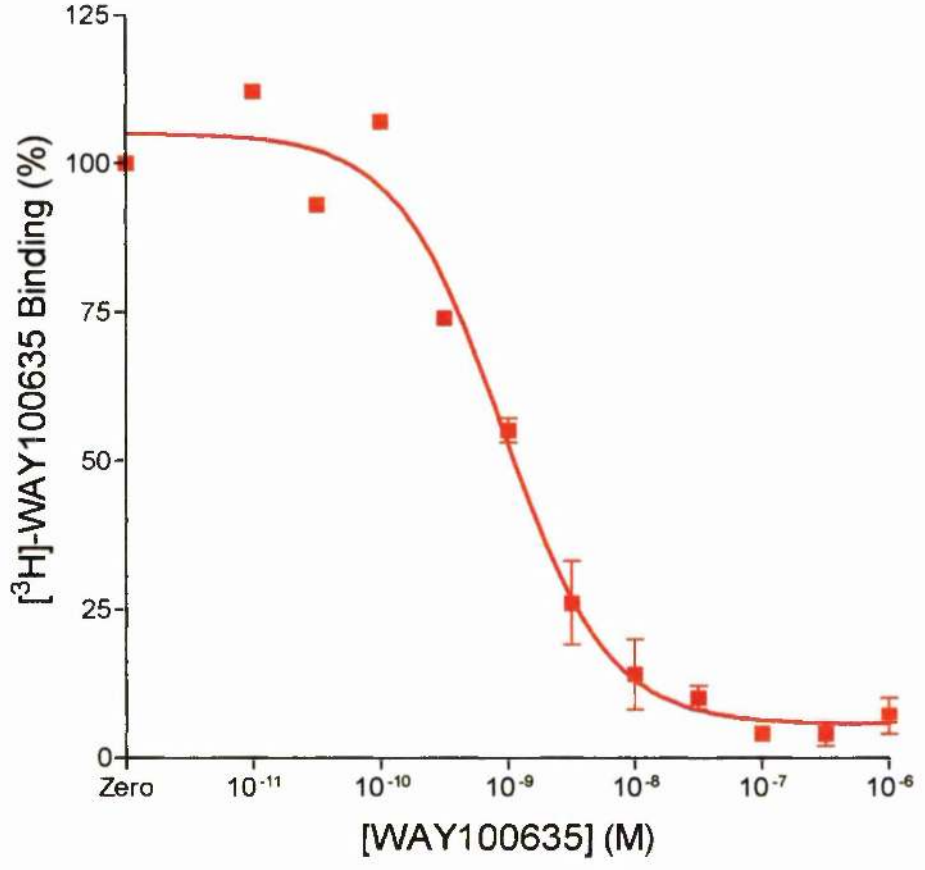
[WAY100635] (M)

B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10-6
[WAY100635] (M)

Figure 6.6 **Saturation analysis of [^{3}H]-WAY100635 binding to the 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I and 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I fusion proteins. Lack of effect of RGS1**

Membranes were prepared from the cells stably expressing the 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I (Panel A) and 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I (Panel B) fusion proteins and saturation binding assays carried out using the 5-HT$_{1A}$ receptor selective antagonist [^{3}H]-WAY100635 in the absence and presence of RGS1 (1μM). Non-specific binding was determined using 10μM WAY100635. Using the data analysis package Graphpad Prism, nonlinear regression analysis of the data fitted a single site binding site, determining total number of binding sites (B_{max}) and the equilibrium dissociation constant (K_d).

Panel A

■ 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I ptox

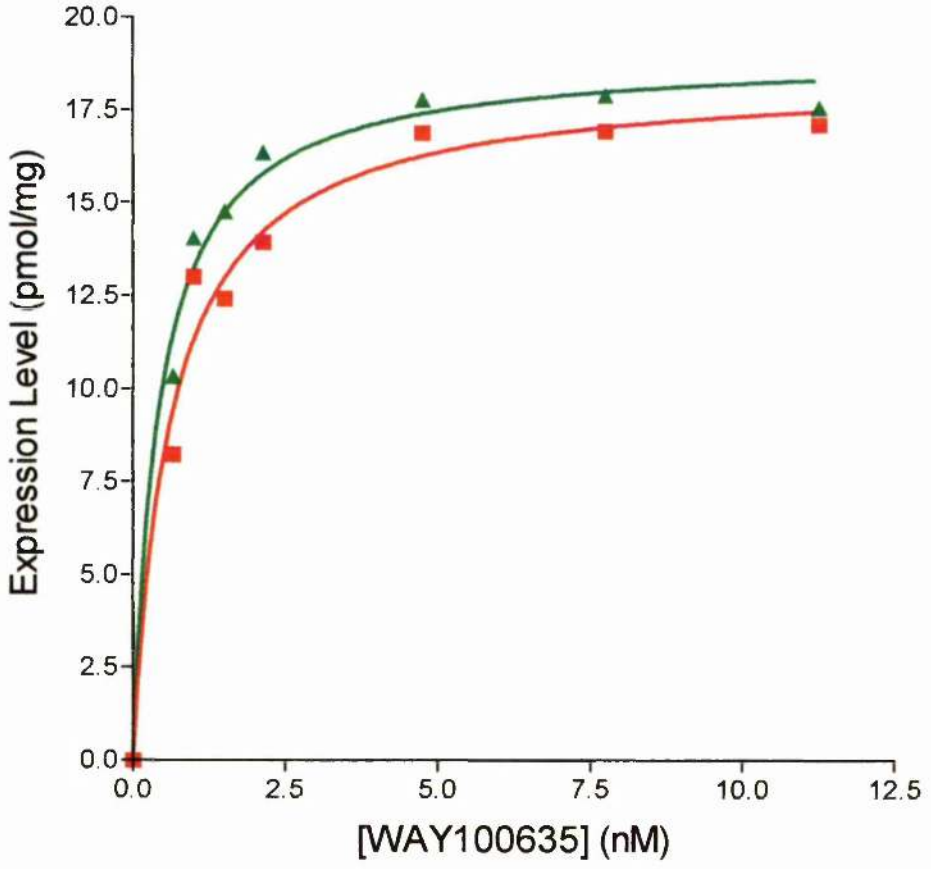
▲ 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I ptox + RGS1 (1μM)

Panel B

■ 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I ptox

▲ 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I ptox + RGS1 (1μM)

A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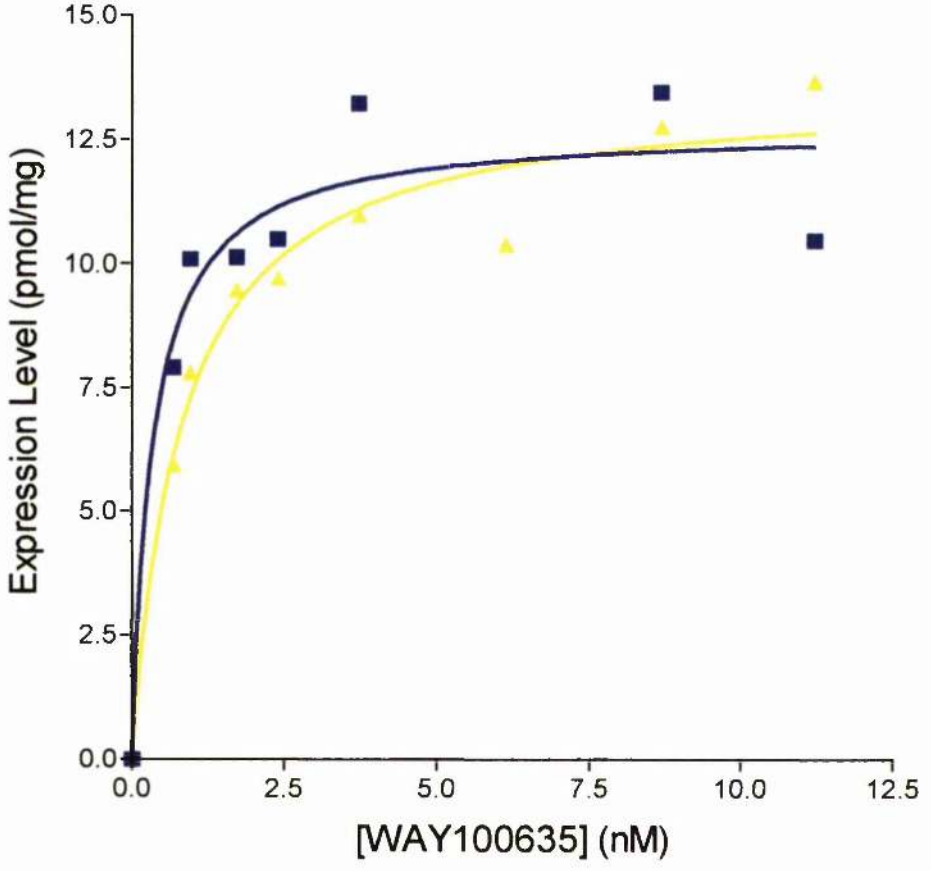
10.0
12.5
[WAY100635] (nM)

B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7.5
10.0
12.5
[WAY100635] (nM)

Figure 6.7 **Panel A: Effects of increasing concentrations of spiperone on the high affinity GTPase activity of membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by spiperone for [^{3}H]-WAY100635 binding to the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of spiperone (1nM – 100μM) in the absence and presence of RGS1 (1μM).

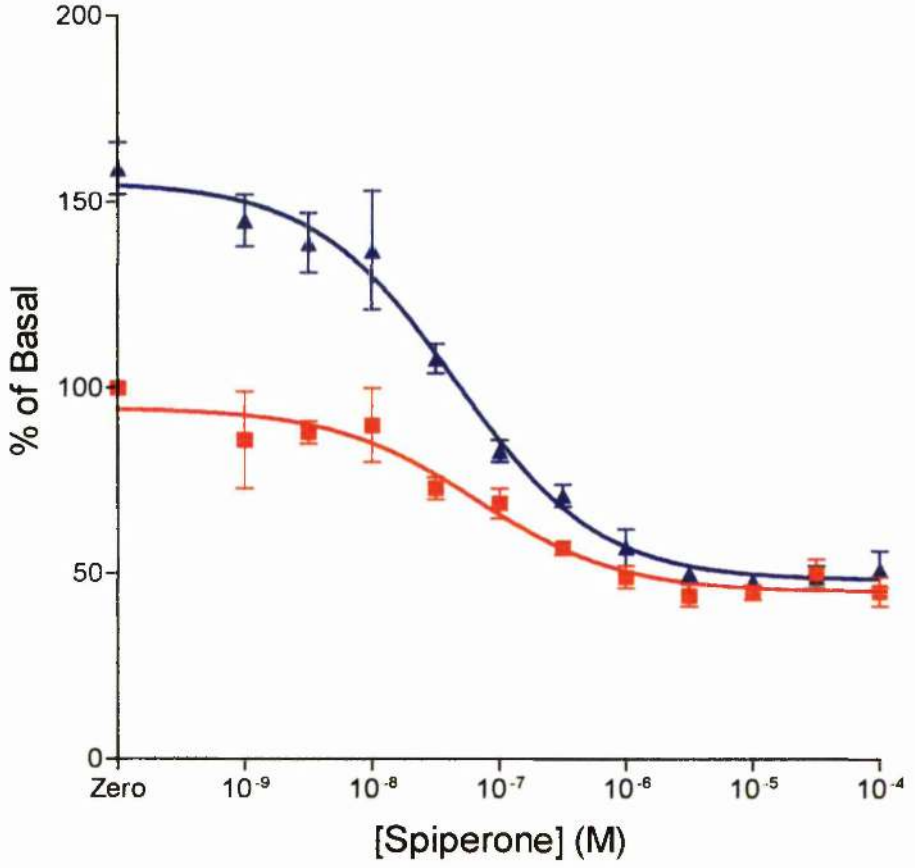
Spiperone

Spiperone + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of spiperone expressed as a percentage of radioligand binding in the absence of spiperone.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A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[Spiperone] (M)

B

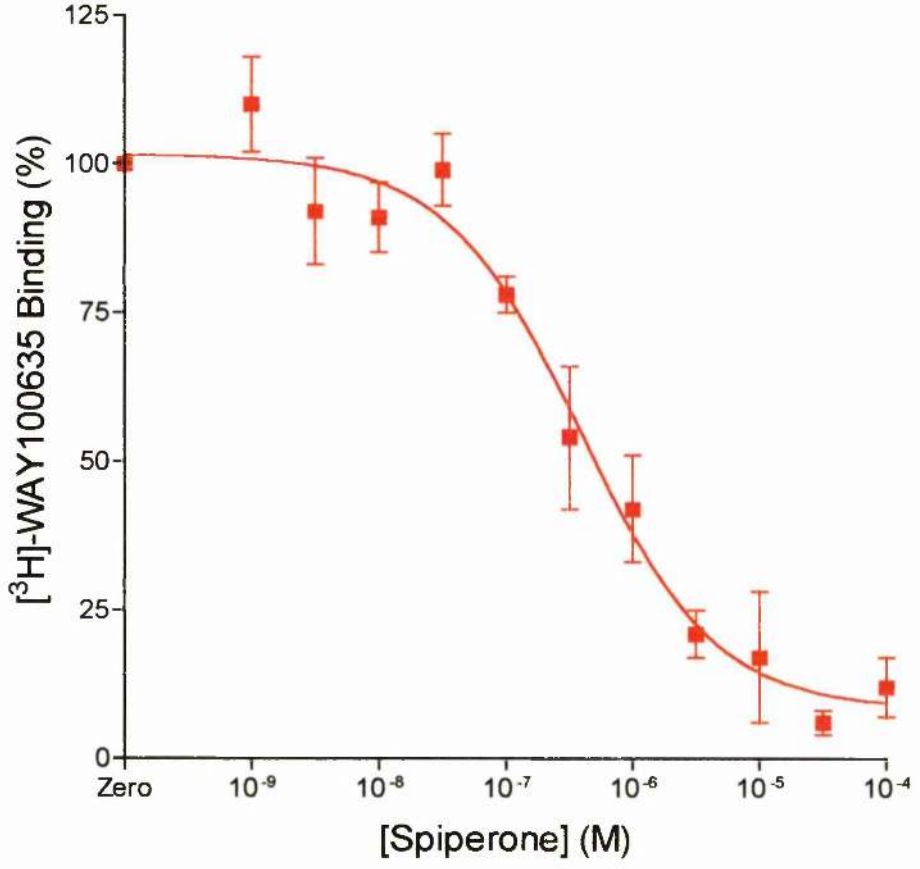

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Spiperone] (M)

Figure 6.8 **Panel A: Effects of increasing concentrations of spiperone on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by spiperone for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of spiperone (1nM – 100μM) in the absence and presence of RGS1 (1μM).

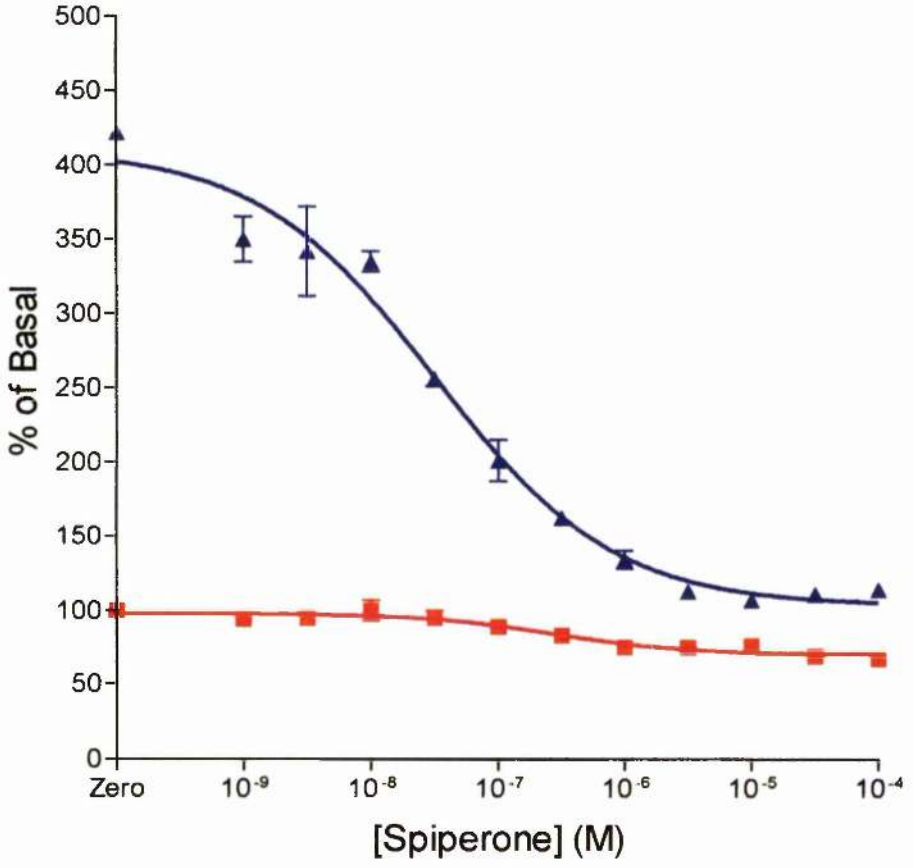
Spiperone

Spiperone + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of spiperone expressed as a percentage of radioligand binding in the absence of spiperone.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


500
450
400
350
300
250
200
150
100
50
0
% of Basal
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Spiperone] (M)

B

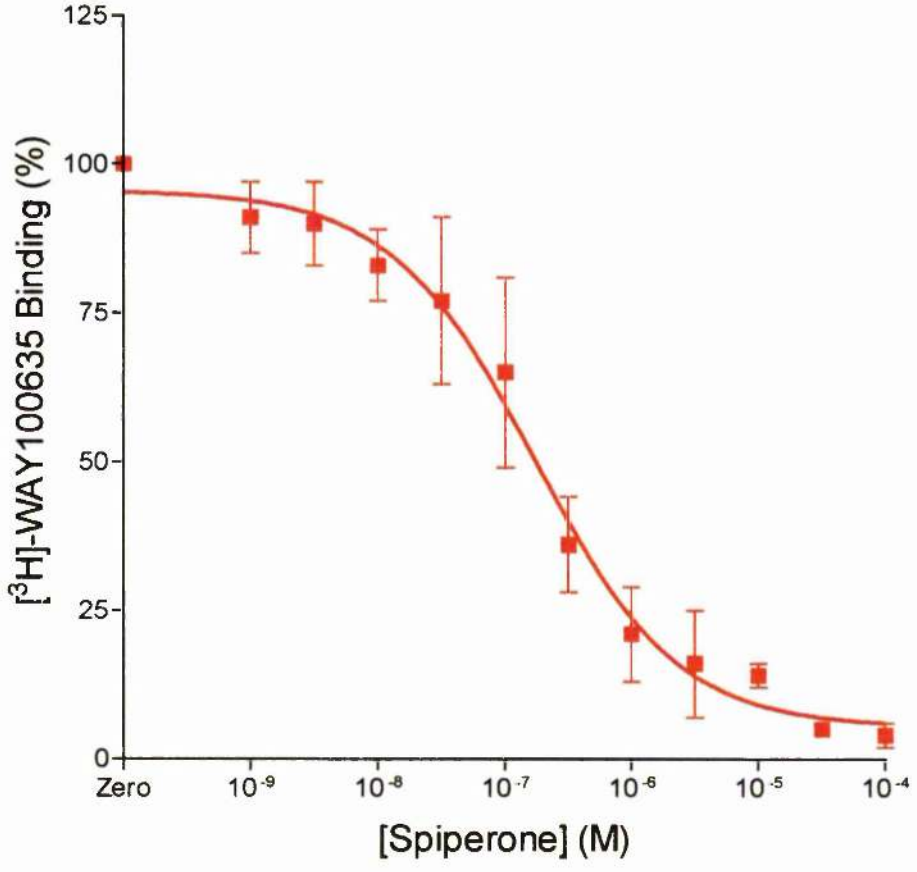

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Spiperone] (M)

Figure 6.9 **Competition by spiperone for [^{3}H]-WAY100635 binding to the 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I (Panel A) and 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I (Panel B) fusion proteins in the absence and presence of RGS1**

Binding of [^{3}H]-WAY100635 in the absence and presence of RGS1 (1μM) to the 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I and 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I fusion proteins in the presence of increasing concentrations of spiperone (1nM – 100μM) expressed as a percentage of radioligand binding in the absence of spiperone.

Data are means of triplicate determinations and are representative of at least three similar experiments.

Panel A

■ 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I ptox

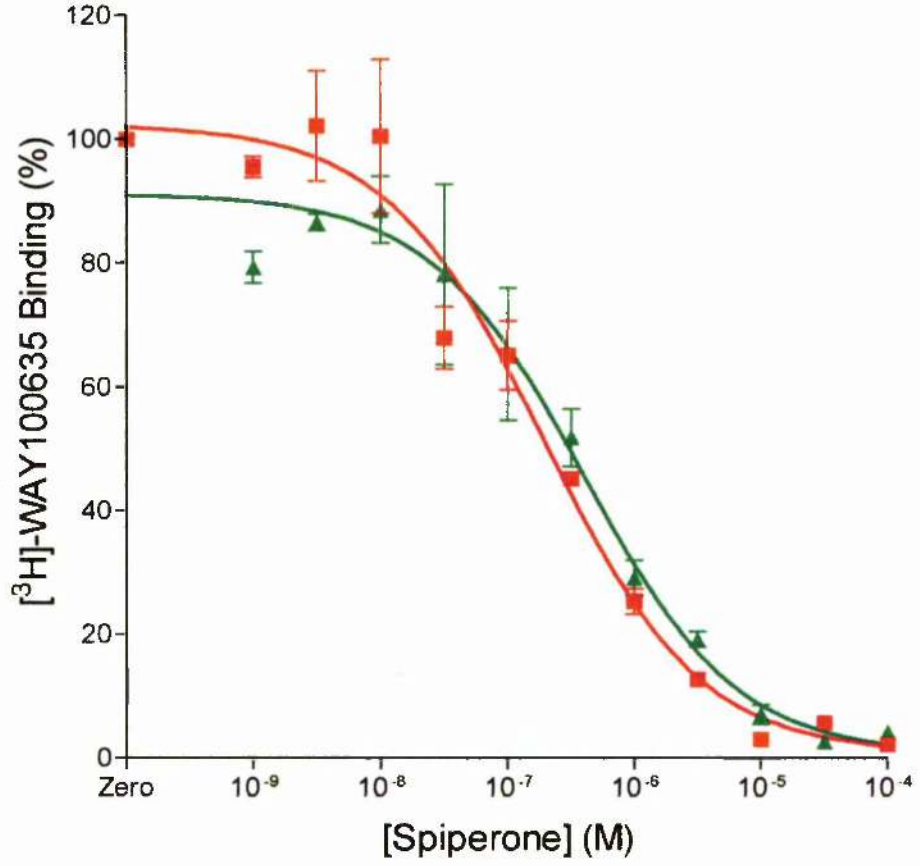
▲ 5-HT$_{1A}$/G$_{i1\alpha}$C^{351}I ptox + RGS1 (1μM)

Panel B

■ 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I ptox

▲ 5-HT$_{1A}$/G$_{o1\alpha}$C^{351}I ptox + RGS1 (1μM)

A


120
100
80
60
40
20
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Spiperone] (M)

B

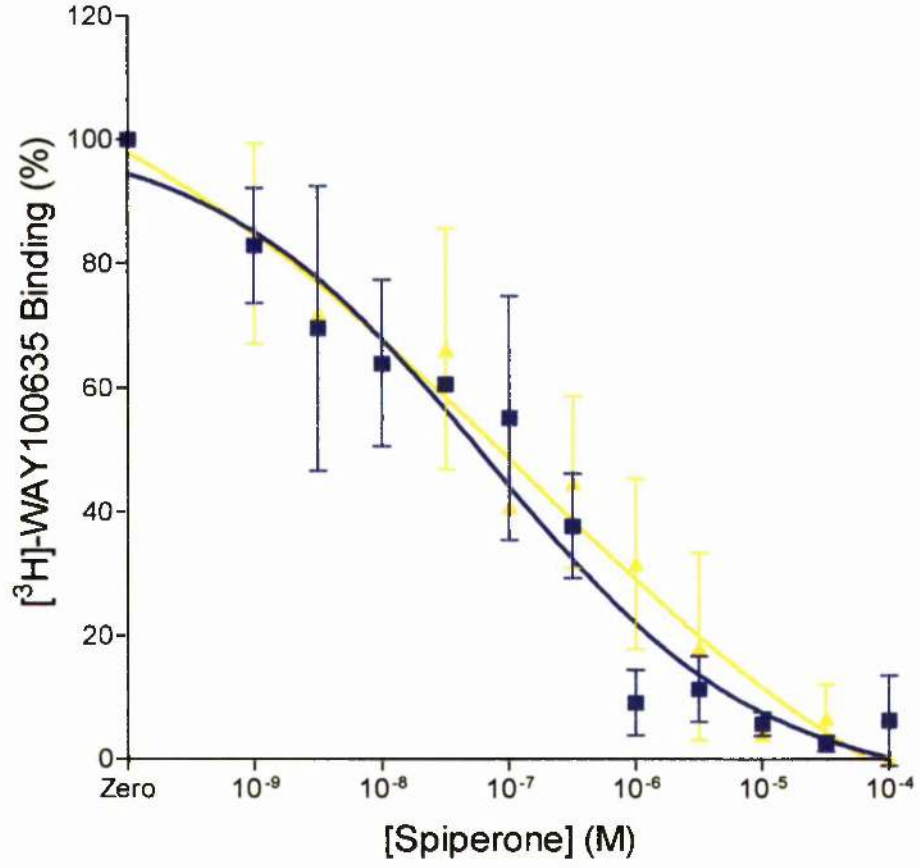

120
100
80
60
40
20
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Spiperone] (M)

Figure 6.10 **Enzyme kinetic analysis of the effects of 5-HT and spiperone on high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the absence and presence of RGS1**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{i1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. 5-HT and spiperone effects on GTPase activity were measured in the presence and absence of RGS1 (1μM). Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.


Basal


5-HT

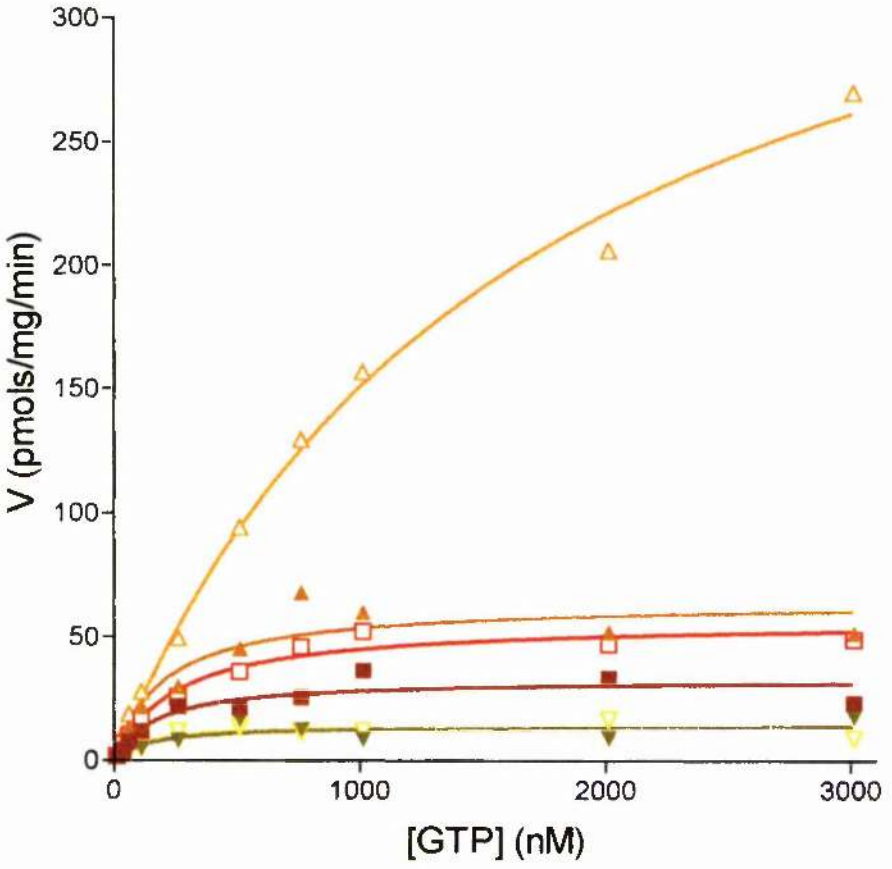

Spiperone


RGS1 (1μM)


5-HT + RGS1

Spiperone + RGS1

A


300
250
200
150
100
50
0
V (pmols/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

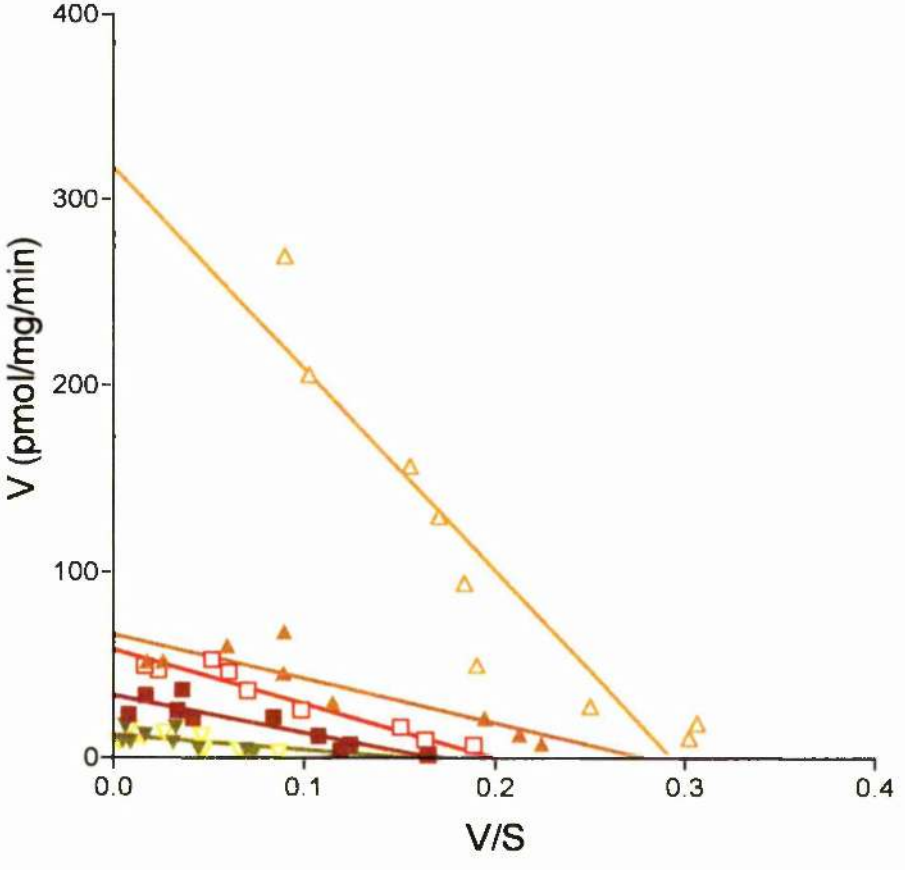
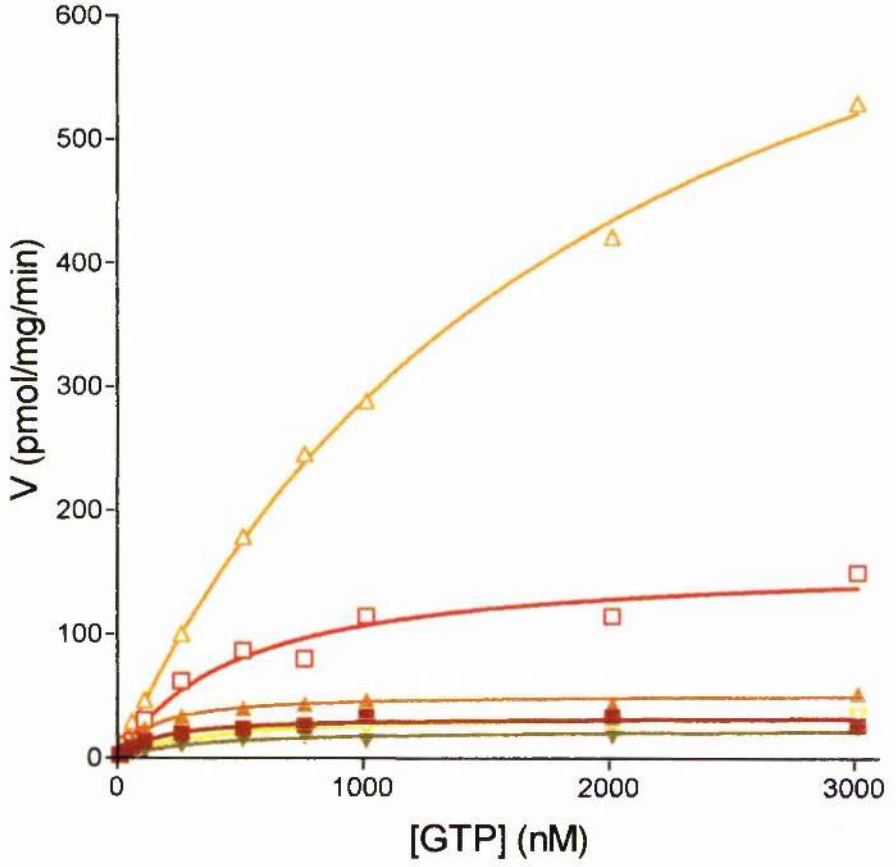

400
300
200
100
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
V/S

Figure 6.11 **Enzyme kinetic analysis of the effects of 5-HT and spiperone on high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the absence and presence of RGS1**

Panel A: GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}G_{o1\alpha}C^{351}I$ fusion protein with increasing concentrations of GTP as substrate. 5-HT and spiperone effects on GTPase activity were measured in the presence and absence of RGS1 (1μM). Panel B: The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max}.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


600
500
400
300
200
100
0
V (pmol/mg/min)
0
1000
2000
3000
[GTP] (nM)

B

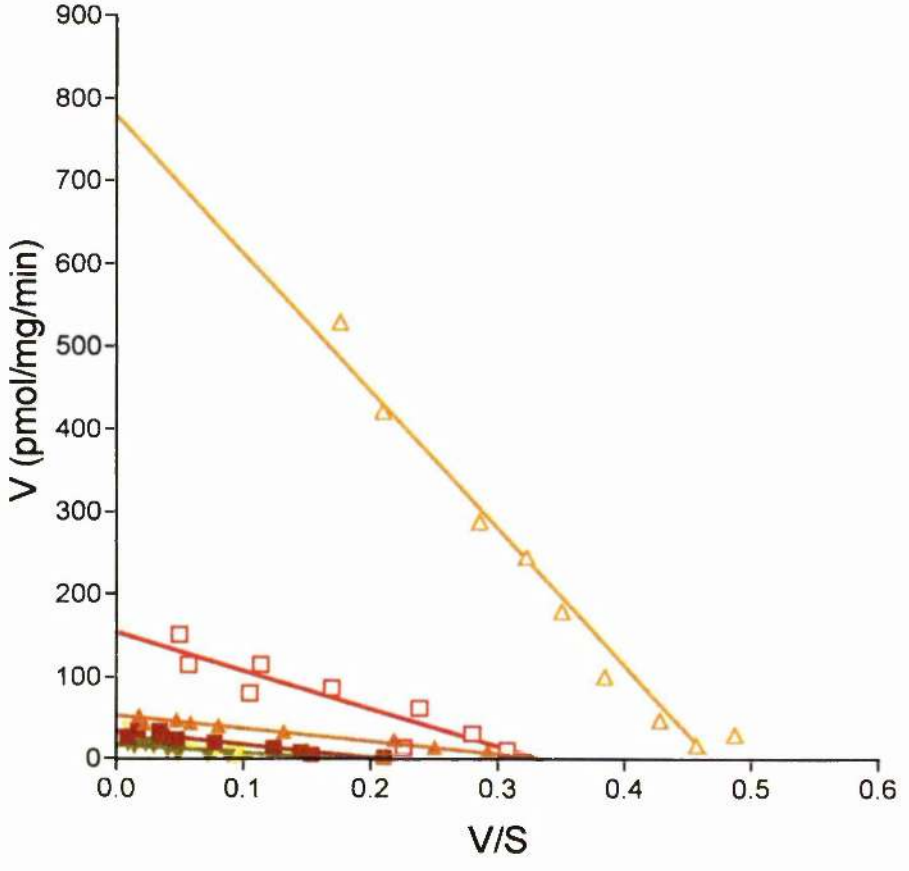

900
800
700
600
500
400
300
200
100
0
V (pmol/mg/min)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
V/S

Table 6.3 **Summary of V_{max} and K_m values from enzyme kinetic analysis for the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins in the presence and absence of both spiperone and RGS1 protein**

The effects of spiperone on basal and RGS1 (1μM) stimulated GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins were measured in terms of V_{max} and K_m for GTP.
Data are means of triplicate determinations and are representative of at least three similar experiments.

	5-$HT_{1A}/G_{i1\alpha}C^{351}I$		5-$HT_{1A}/G_{o1\alpha}C^{351}I$	
Treatment	V_{max}(nM)	K_m (nM)	V_{max} (nM)	K_m (nM)
Basal	33+/-3.2	151+/-28	23+/-0.9	145+/-11
RGS1 (1μM)	58+/-3.5	292+/-30	13+/-1.5	100+/-26
Spiperone	12+/-1.9	77+/-29	146+/-11	492+/-61
Spiperone + RGS1	13+/-1.5	81+/-23	24+/-2.7	143+/-33

Figure 6.12 **Panel A: Effects of increasing concentrations of methiothepine on the high affinity GTPase activity of membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by methiothepine for [^{3}H]-WAY100635 binding to the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of methiothepine (0.1nM – 10μM) in the absence and presence of RGS1 (1μM).

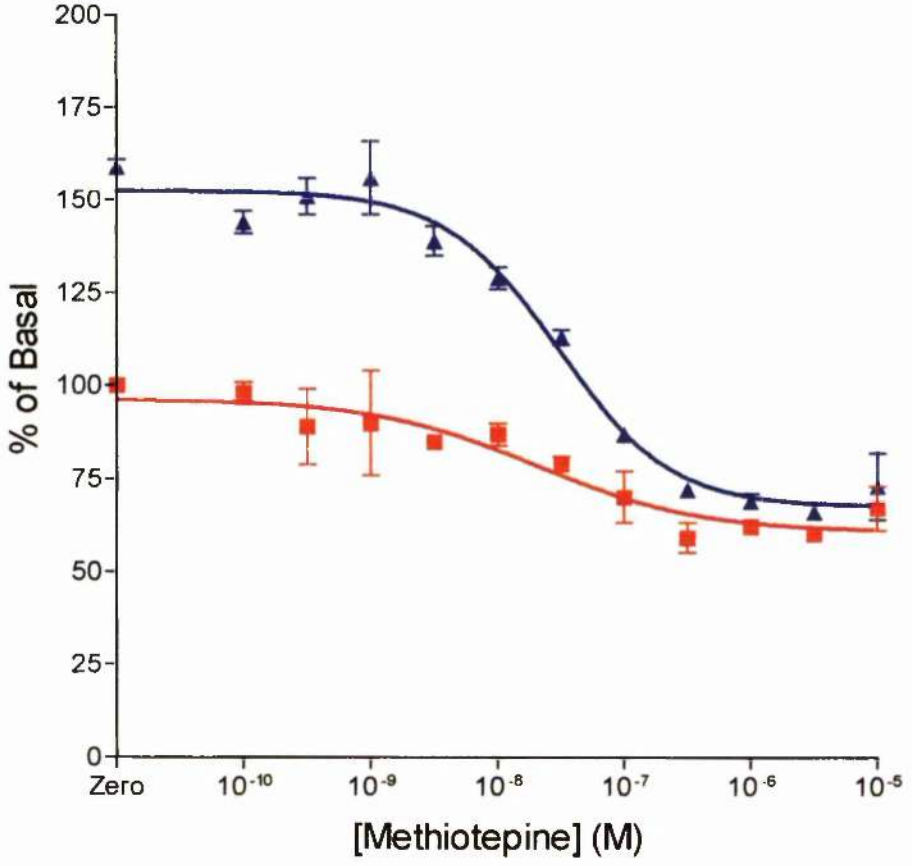
Methiothepine

Methiothepine + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of methiothepine expressed as a percentage of radioligand binding in the absence of methiothepine.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


200
175
150
125
100
75
50
25
0
% of Basal
Zero
10-10
10-9
10-8
10-7
10-6
10-5
[Methiotepine] (M)

B

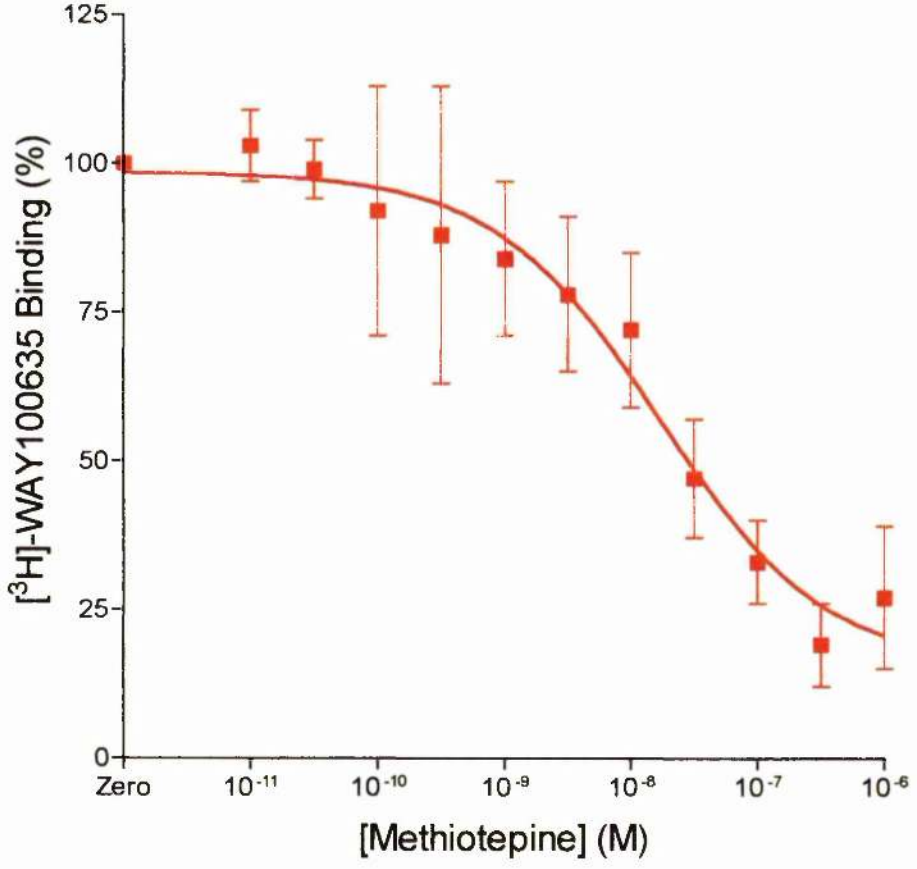

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-11
10-10
10-9
10-8
10-7
10-6
[Methiotepine] (M)

Figure 6.13 **Panel A: Effects of increasing concentrations of methiothepine on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by methiothepine for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of methiothepine (0.1nM – 10μM) in the absence and presence of RGS1 (1μM).

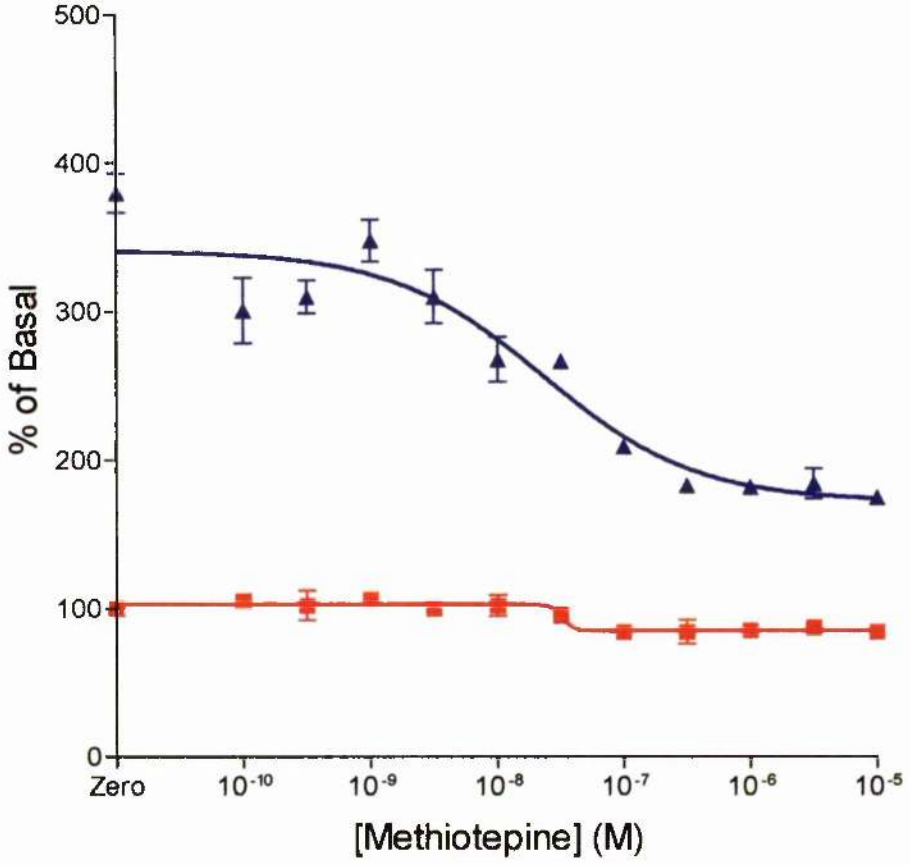
Methiothepine

Methiothepine + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of methiothepine expressed as a percentage of radioligand binding in the absence of methiothepine.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


500
400
300
200
100
0
% of Basal
Zero
10-10
10-9
10-8
10-7
10-6
10-5
[Methiotepine] (M)

B

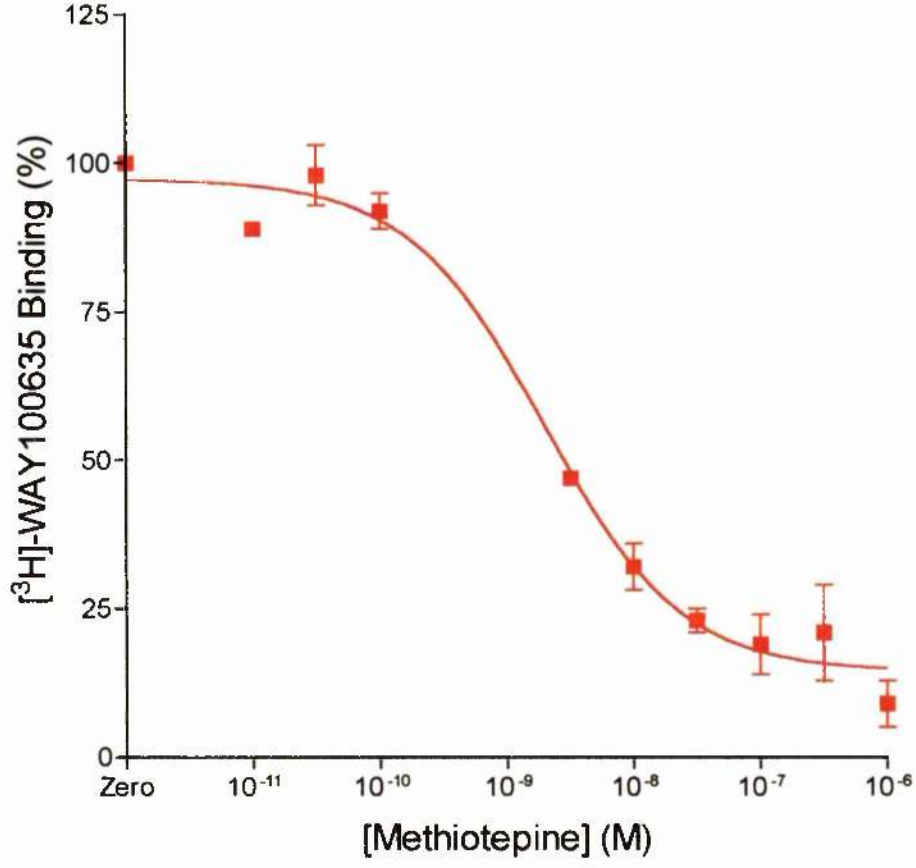

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-11
10-10
10-9
10-8
10-7
10-6
[Methiotepine] (M)

Figure 6.14 **Panel A: Effects of increasing concentrations of chlorpromazine on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by chlorpromazine for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of chlorpromazine (1nM – 100μM) in the absence and presence of RGS1 (1μM).

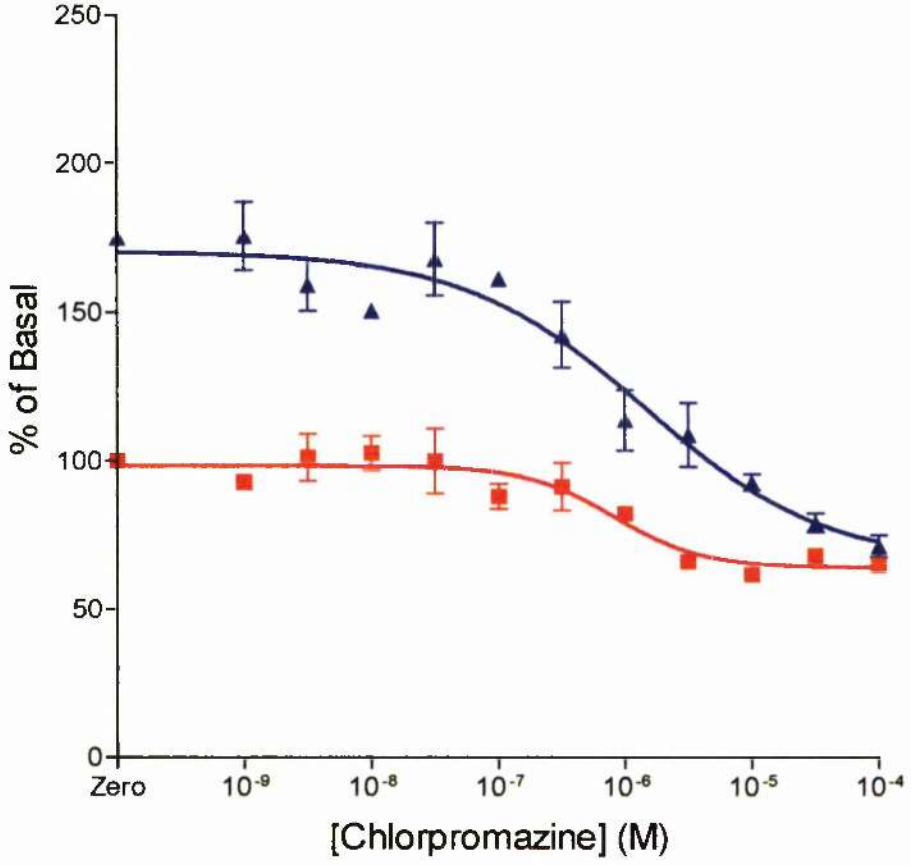
Chlorpromazine

Chlorpromazine + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of chlorpromazine expressed as a percentage of radioligand binding in the absence of chlorpromazine.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


250
200
150
100
50
0
% of Basal
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Chlorpromazine] (M)

B

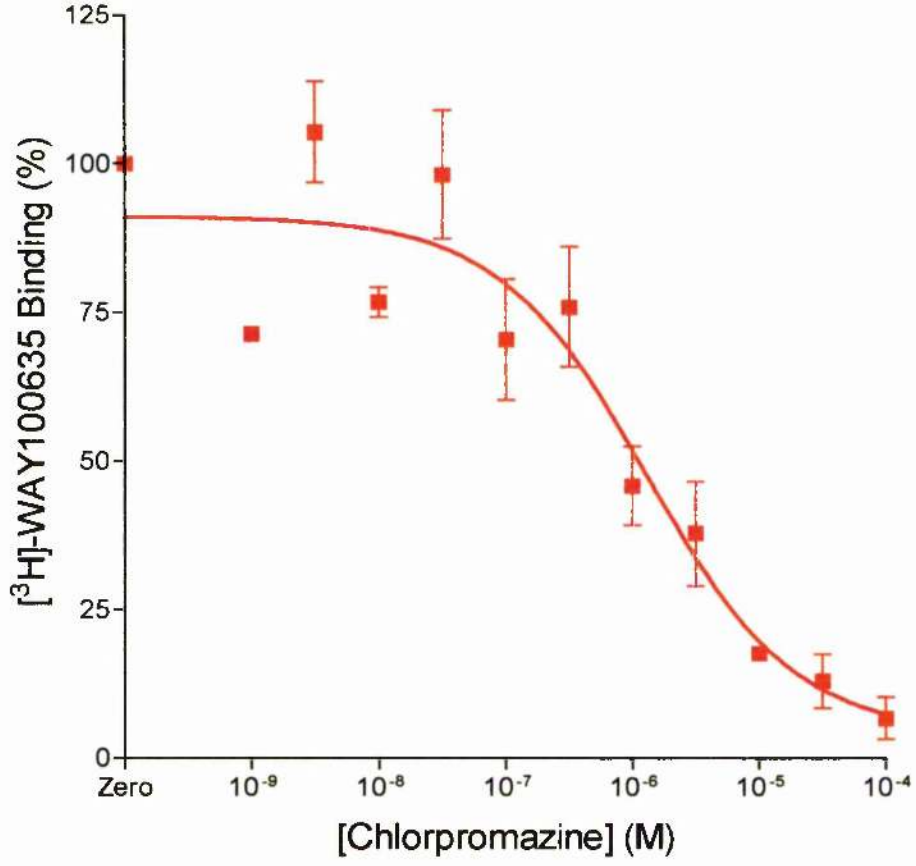

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Chlorpromazine] (M)

Figure 6.15 **Panel A: Effects of increasing concentrations of chlorpromazine on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by chlorpromazine for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of chlorpromazine (1nM – 100μM) in the absence and presence of RGS1 (1μM).

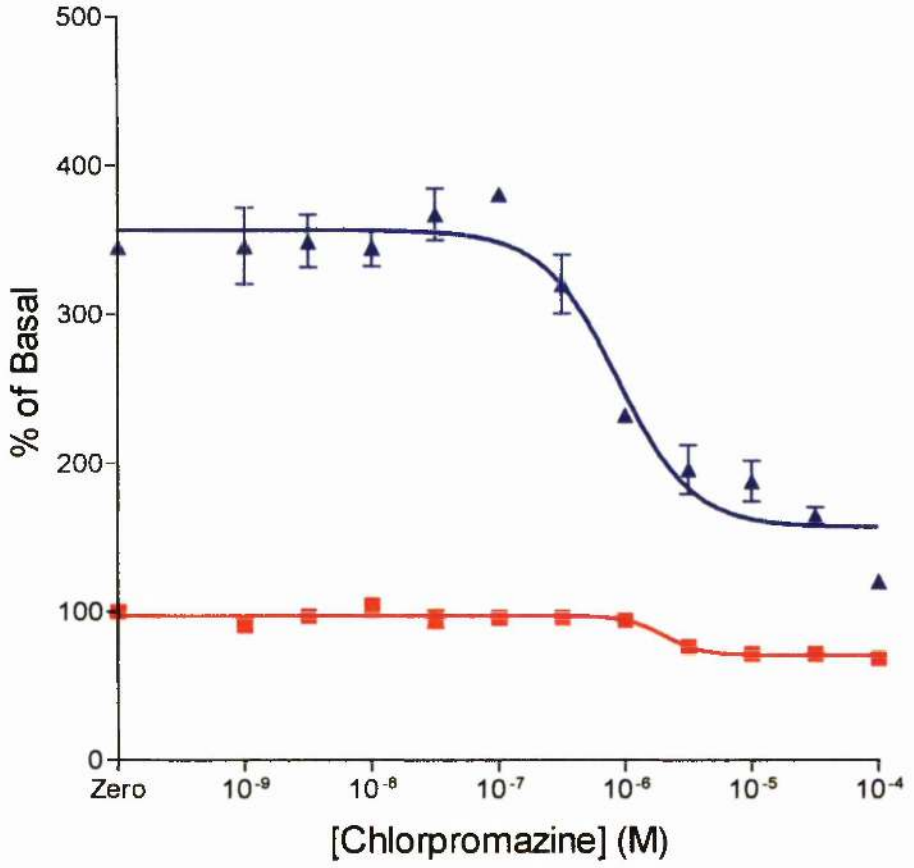
□ Chlorpromazine

▼ Chlorpromazine + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of chlorpromazine expressed as a percentage of radioligand binding in the absence of chlorpromazine.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


500
400
300
200
100
0
% of Basal
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Chlorpromazine] (M)

B

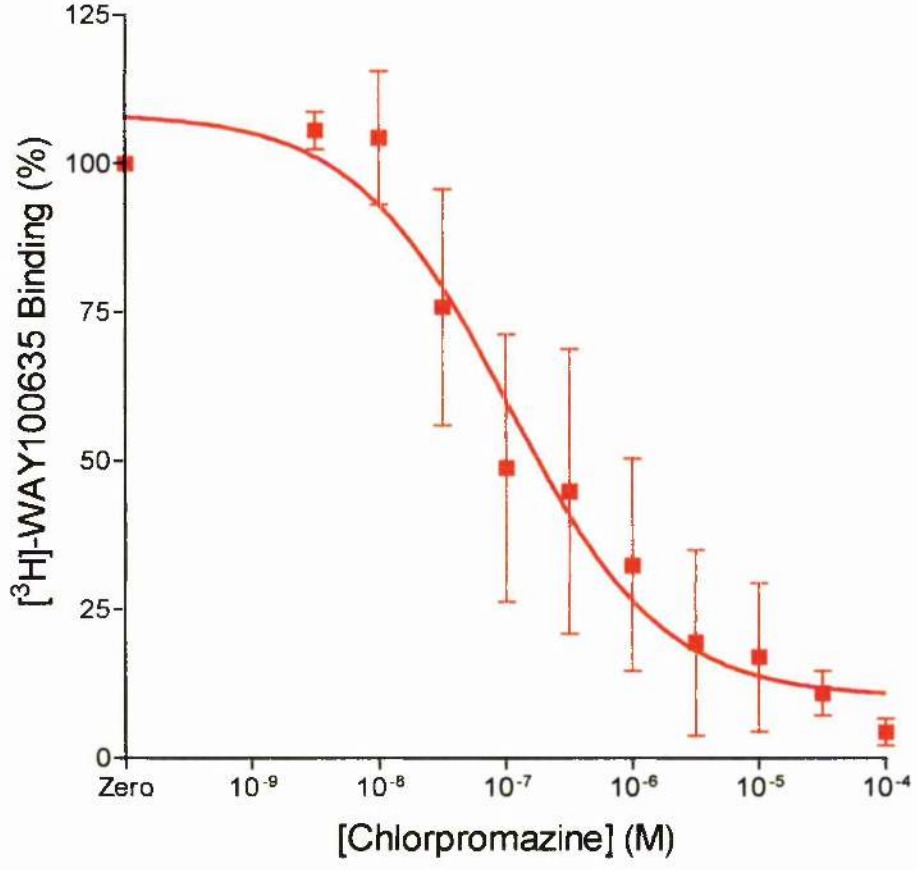

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Chlorpromazine] (M)

Figure 6.16 **Panel A: Effects of increasing concentrations of butaclamol on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by butaclamol for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of butaclamol (1nM – 100μM) in the absence and presence of RGS1 (1μM).

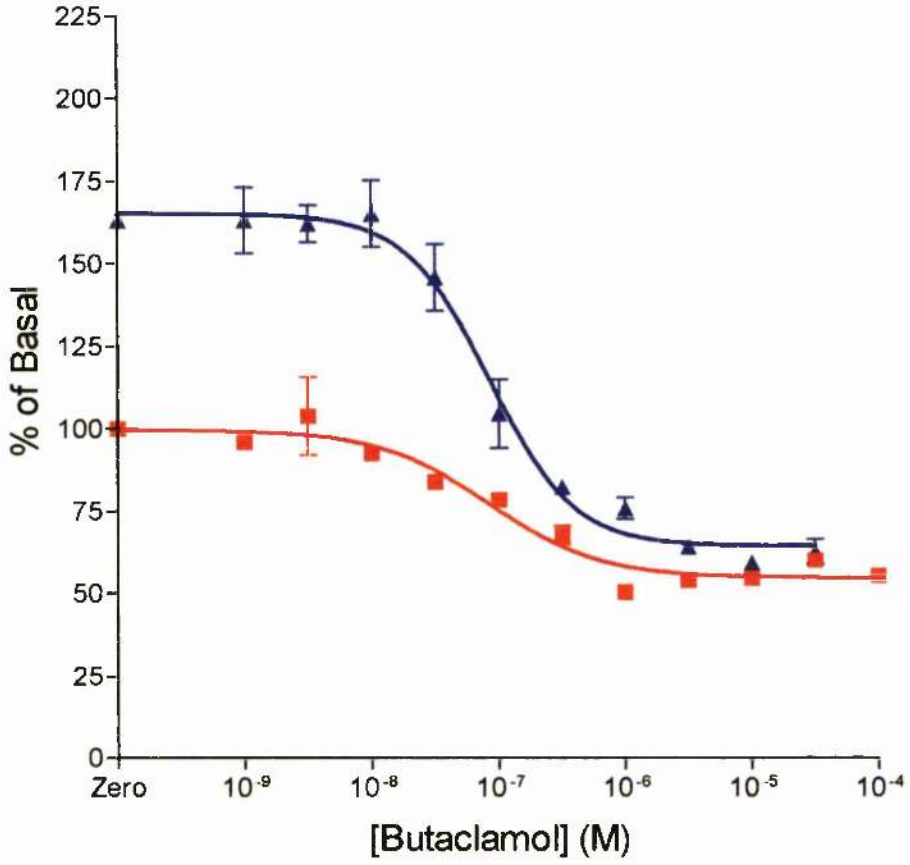
□ Butaclamol

▼ Butaclamol + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of butaclamol expressed as a percentage of radioligand binding in the absence of butaclamol.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


225
200
175
150
125
100
75
50
25
0
% of Basal
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Butaclamol] (M)

B

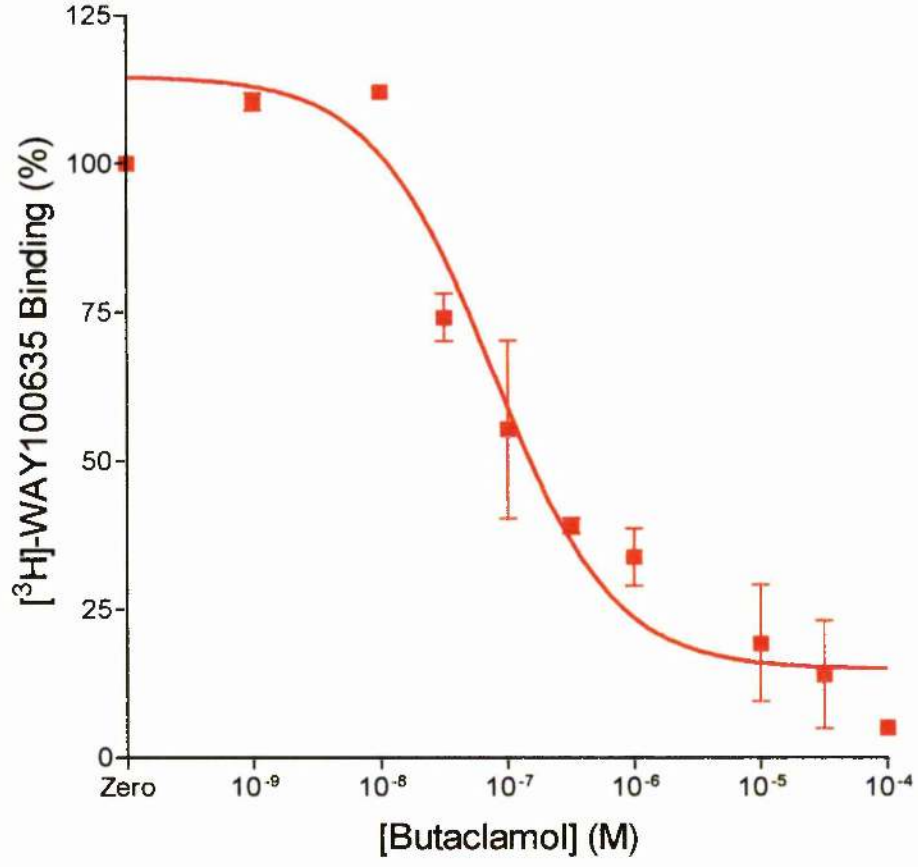

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Butaclamol] (M)

Figure 6.17 **Panel A: Effects of increasing concentrations of butaclamol on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by butaclamol for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of butaclamol (1nM – 100μM) in the absence and presence of RGS1 (1μM).

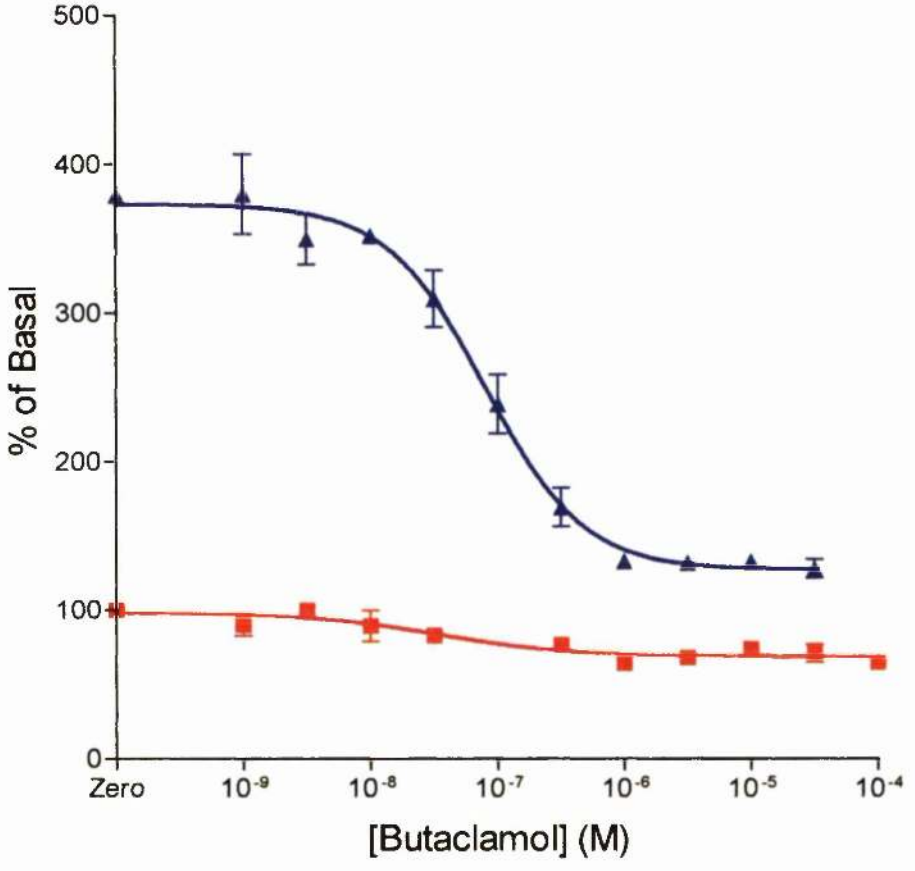
■ Butaclamol

▼ Butaclamol + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of butaclamol expressed as a percentage of radioligand binding in the absence of butaclamol.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


500
400
300
200
100
0
% of Basal
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Butaclamol] (M)

B

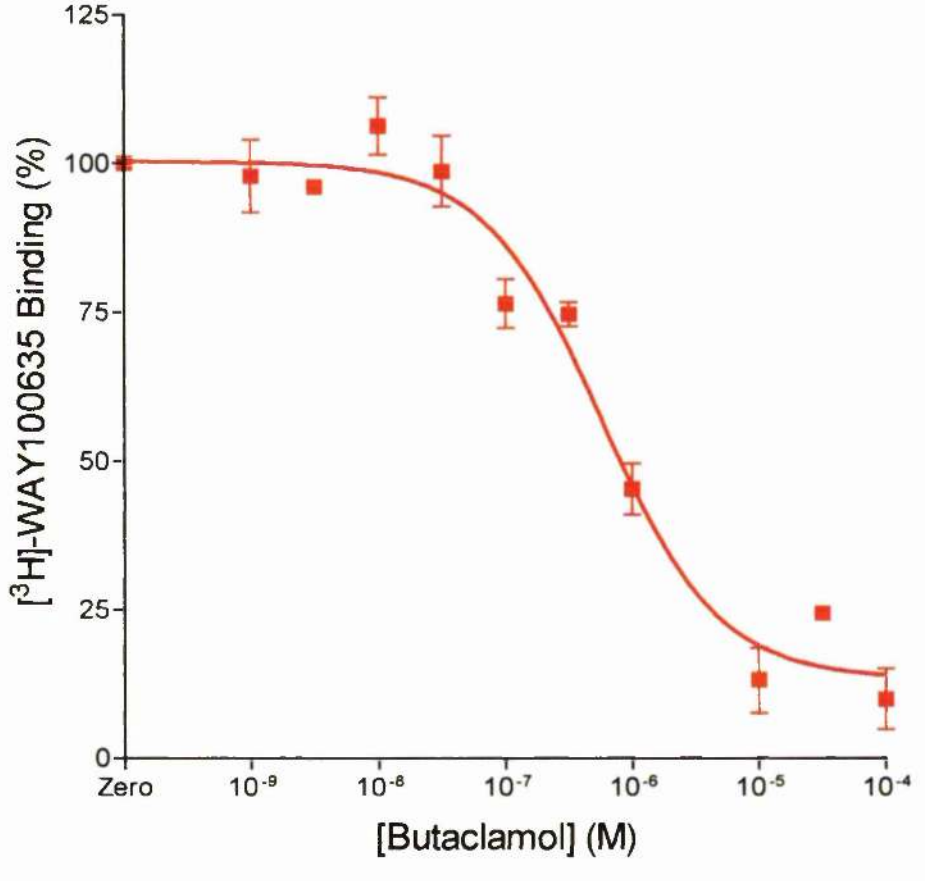

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Butaclamol] (M)

Figure 6.18 **Panel A: Effects of increasing concentrations of thioridazine on the high affinity GTPase activity of membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by thioridazine for [^{3}H]-WAY100635 binding to the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of thioridazine (1nM – 100μM) in the absence and presence of RGS1 (1μM).

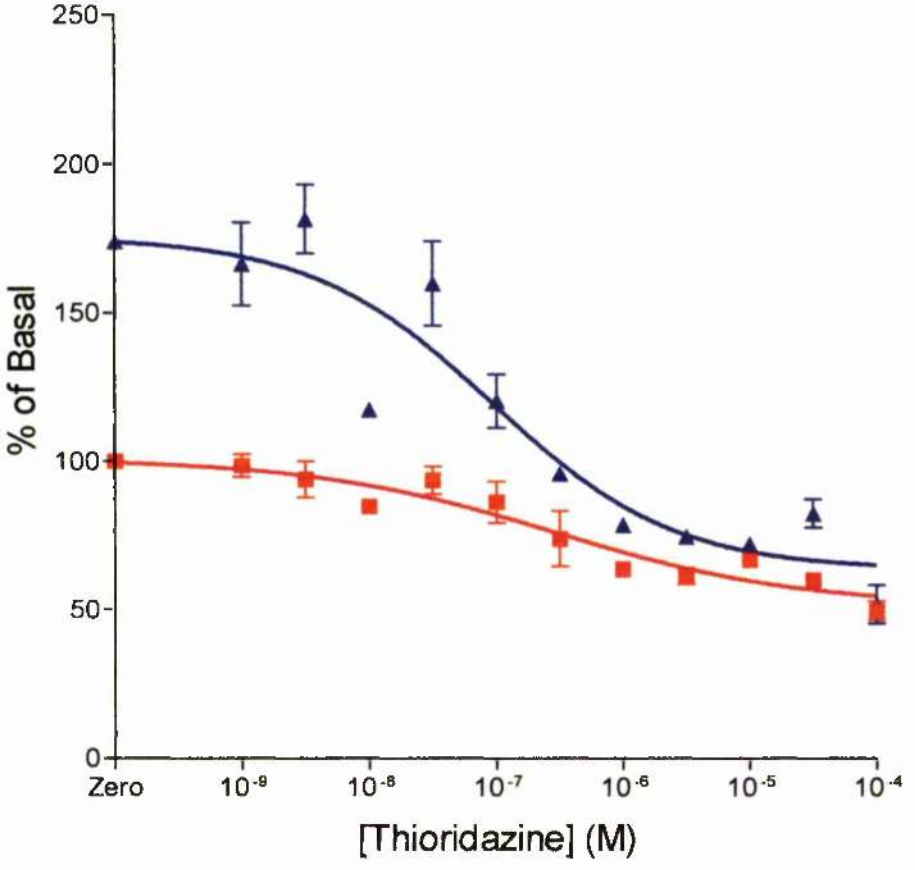
Thioridazine

Thioridazine + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of thioridazine expressed as a percentage of radioligand binding in the absence of thioridazine.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A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200
150
100
50
0
% of Basal
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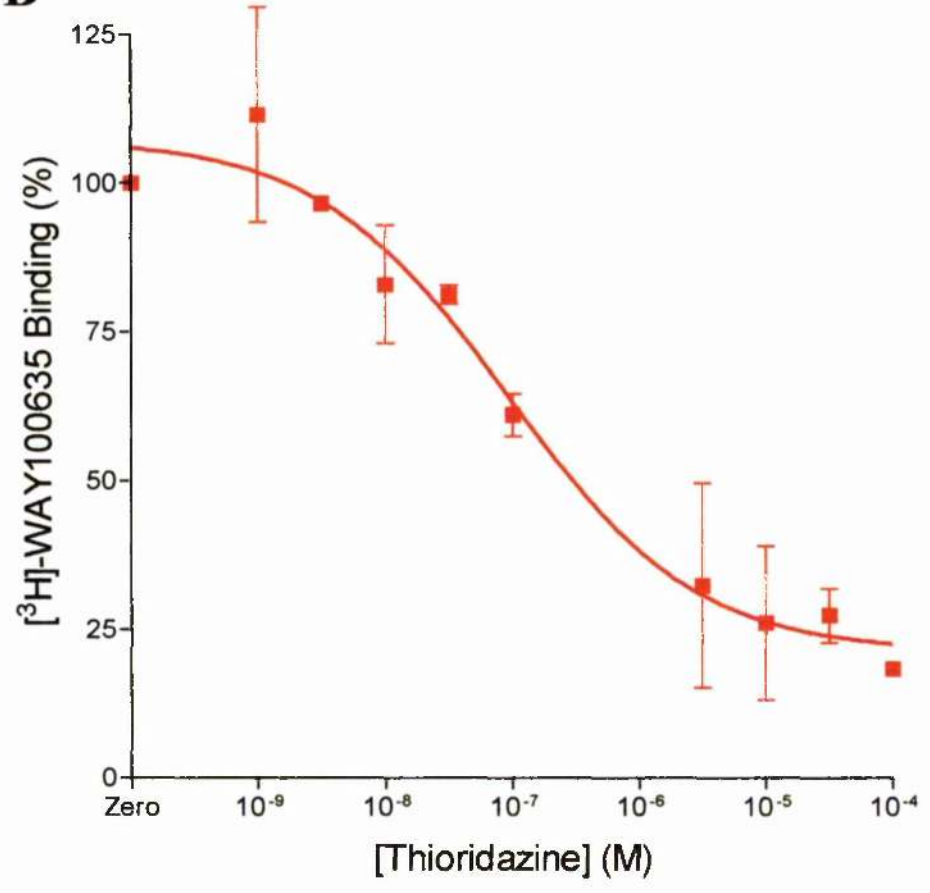
10-9
10-8
10-7
10-6
10-5
10-4
[Thioridazine] (M)

B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75
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[3H]-WAY100635 Binding (%)
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10-9
10-8
10-7
10-6
10-5
10-4
[Thioridazine] (M)

Figure 6.19 **Panel A: Effects of increasing concentrations of thioridazine on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by thioridazine for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of thioridazine (1nM – 100μM) in the absence and presence of RGS1 (1μM).

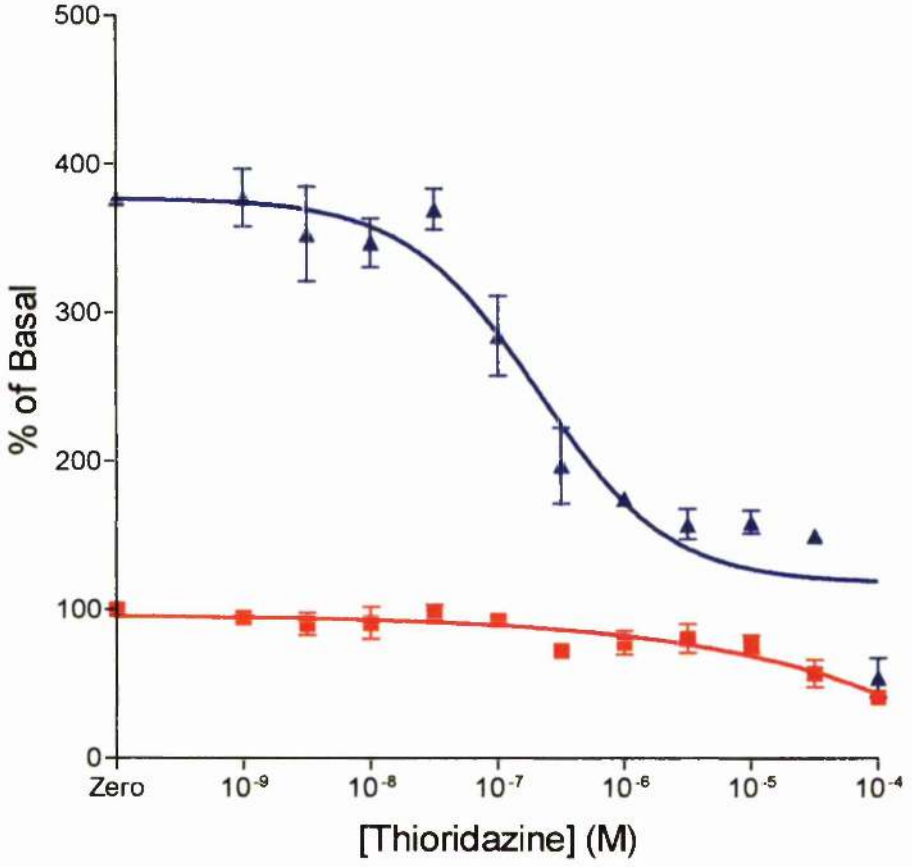
Thioridazine

Thioridazine + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of thioridazine expressed as a percentage of radioligand binding in the absence of thioridazine.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A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400
300
200
100
0
% of Basal
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Thioridazine] (M)

B

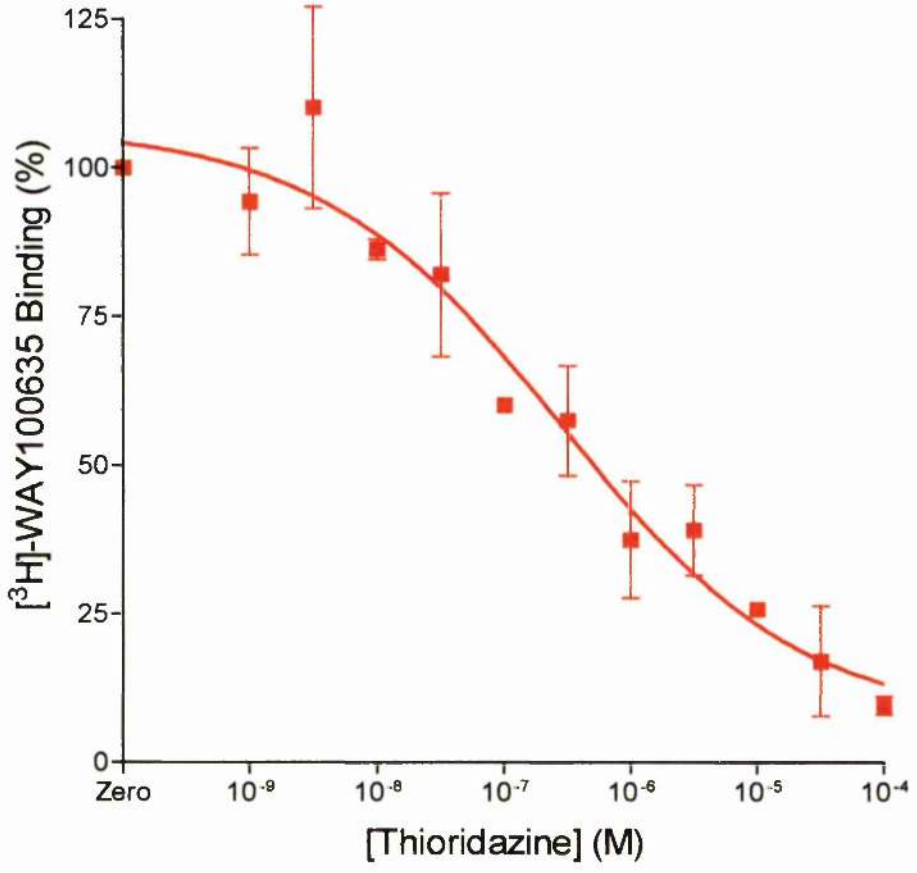

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Thioridazine] (M)

Figure 6.20 **Panel A: Effects of increasing concentrations of haloperidol on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by haloperidol for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of haloperidol (1nM – 100μM) in the absence and presence of RGS1 (1μM).

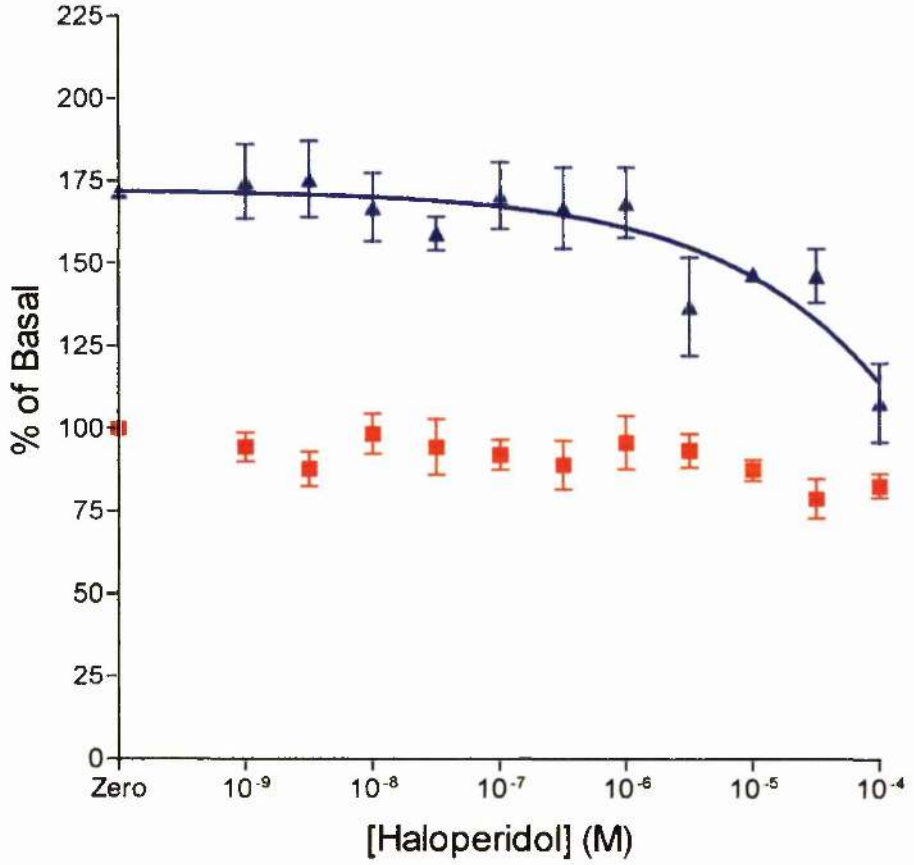
□ Haloperidol

▼ Haloperidol + RGS1 (1μM)

Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of haloperidol expressed as a percentage of radioligand binding in the absence of haloperidol.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A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200
175
150
125
100
75
50
25
0
% of Basal
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Haloperidol] (M)

B

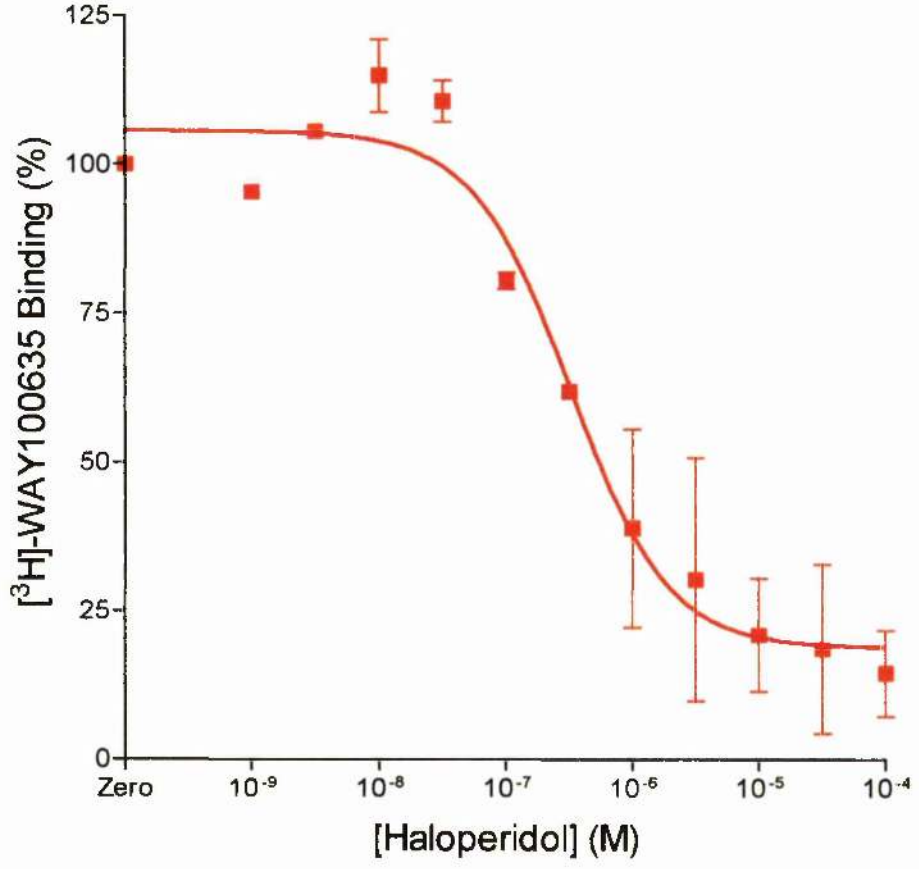

125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Haloperidol] (M)

Figure 6.21 **Panel A: Effects of increasing concentrations of haloperidol on the high affinity GTPase activity of membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence and absence of RGS1. Panel B: Competition by haloperidol for [^{3}H]-WAY100635 binding to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein.**

Panel A: High affinity GTPase activity in pertussis toxin treated membranes expressing the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein expressed as percentage stimulation of basal GTPase activity in the absence of RGS1. Effects of increasing concentrations of haloperidol (1nM – 100μM) in the absence and presence of RGS1 (1μM).

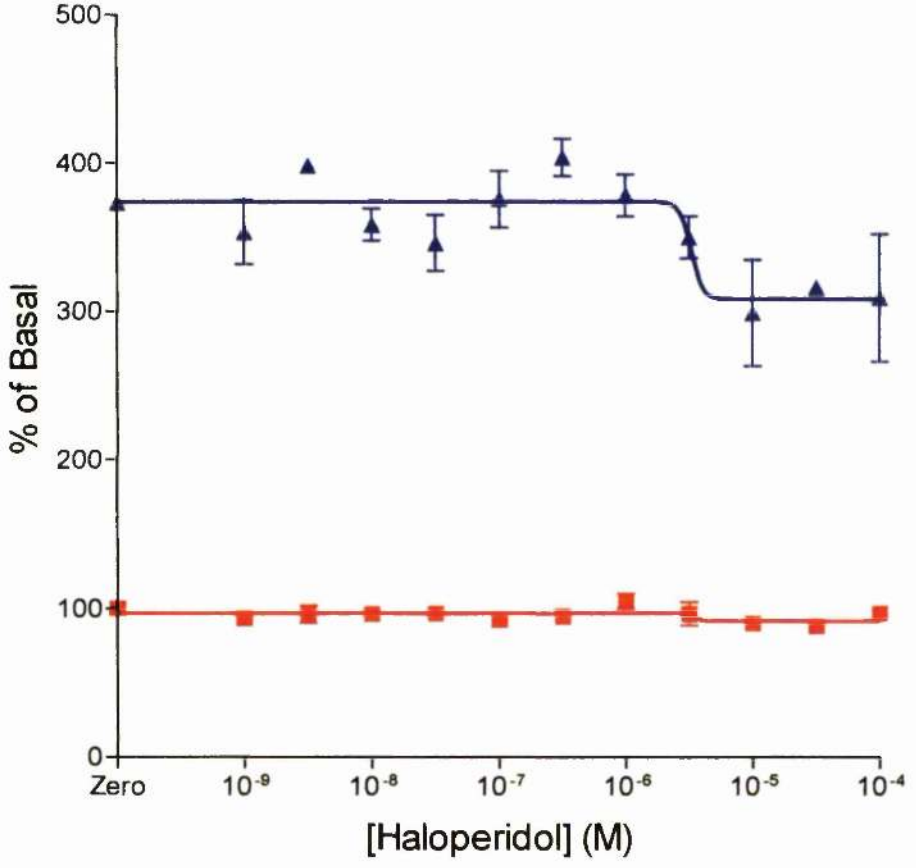
■ Haloperidol

▼ Haloperidol + RGS1 (1μM)

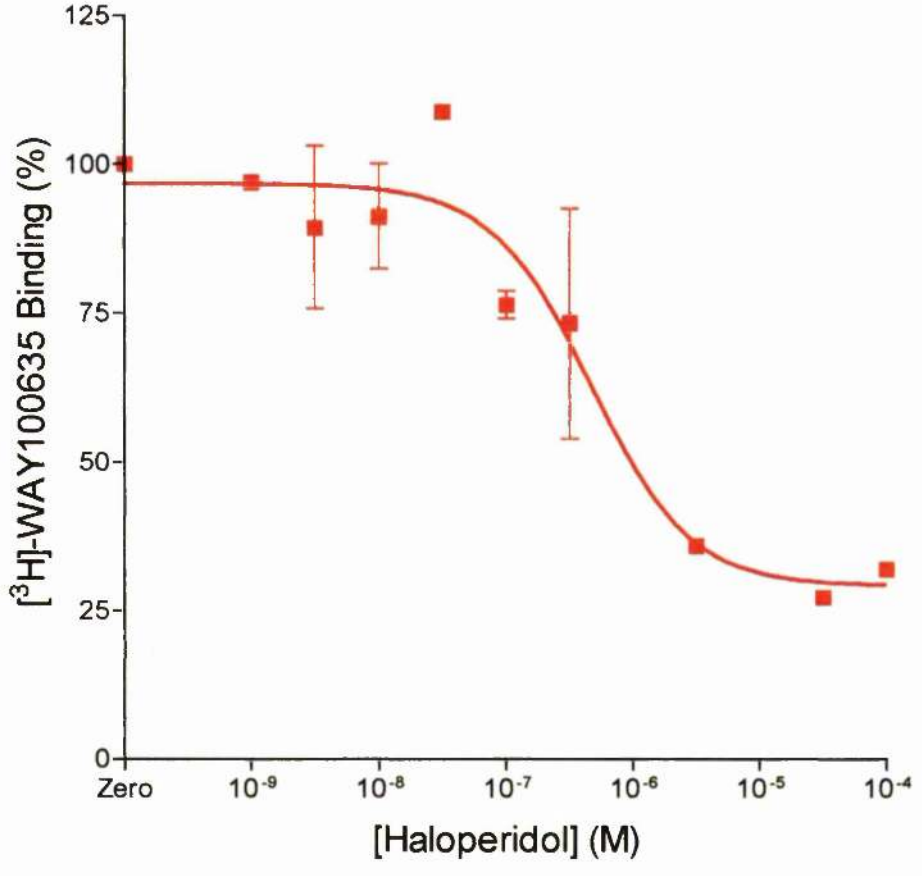
Panel B: Specific binding of [^{3}H]-WAY100635 to the 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion protein in the presence of increasing concentrations of haloperidol expressed as a percentage of radioligand binding in the absence of haloperidol.

Data are means of triplicate determinations and are representative of at least three similar experiments.

A


500
400
300
200
100
0
% of Basal
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Haloperidol] (M)

B


125
100
75
50
25
0
[3H]-WAY100635 Binding (%)
Zero
10-9
10-8
10-7
10-6
10-5
10-4
[Haloperidol] (M)

6.2 Discussion

From a sceptical start, inverse agonists have redefined the classification of a large number of drugs. Those previously classified simply as antagonists have now been shown in many cases to display inverse agonist activity. For example, a number of drugs were originally designed as antagonists for the histamine H_2 receptor, cimetadine, ranitidine and famotidine, have been shown to be inverse agonists. Their use results in the up regulation of H_2 receptors of up to 180% in mammalian cell lines (Alewijnse *et al.*, 1998; Smit *et al.*, 1996). Although a correlation between the level of receptor upregulation and withdrawal effects has not been found it has been suggested that receptor upregulation by these H_2 receptor inverse agonists may lead to drug tolerance in ulcer treatment (Leurs *et al.*, 1998). Distinction between inverse agonists and ligands acting as neutral antagonists may therefore be important in development of future drug therapies.

Constitutive activity was first described for a GPCR in 1989 for the δ opioid receptor in NG108-15 neuroblastoma-glioma cells (Costa and Herz 1989) and later for overexpressed α_{1B} and β_2 adrenoceptors (Lefkowitz *et al.*, 1993). Many ligands previously described as antagonists were subsequently re-classified following demonstration of their inverse activity (Milligan *et al*, 1995; Kenakin, 1996; Leff, 1997).

The activity of a receptor can be very simple classed into two states, G protein uncoupled (R) and G protein coupled (R*), where in the R* form the receptor has increased affinity for agonists and a higher basal level of activity. Constitutive activity can be induced by a number of methods. Firstly and most simply, the receptor can be over expressed in a system. By increasing receptor number, there is an increase in the number of copies of R* receptors (Chidiac *et al.*, 1994; MacEwan and Milligan, 1996; Smit *et al.*, 1996). Secondly, certain mutations in the DRY motif, a highly conserved region at the cytosolic end of the transmembrane domain 3, can lead to increased G protein coupling, possibly by exposing residues in the intracellular domains of the GPCR that are normally only exposed following agonist binding (Scheer *et al.*, 1996). Thirdly, fusion proteins between the GPCR and the G_α subunits of inhibitory G proteins can also increase the constitutive activity of the receptor (Kellett *et al.*, 1999). This increase in constitutive activity is enhanced by mutation of Cys^{351} in the C terminal region of the α subunit to more hydrophobic

amino acids. Bahia *et al.* (1998) mutated Cys^{351} to every other naturally occurring amino acid and found a strong correlation between the hydrophobicity of the residue and its affinity for the α_{2A}-adrenoceptor.

The data in this chapter demonstrates a clear role for both receptor-G protein mutant fusion proteins and the use of RGS proteins in distinguishing between neutral antagonists and inverse agonists. They can also help to provide useful pharmacological information on the potency of inverse agonists at decreasing basal receptor activity. Previously, data on inverse agonists has been generated from systems where the receptor has been overexpressed or mutated to increase constitutive activity. This results in a less than natural system, particularly in the case of the mutant GPCRs and only provides comparative data for each expression system and assay used. Using fusion proteins between the GPCR and G protein allows for the generation of much more accurate data with a WT receptor where any mutations used will be in the G_α protein. The 1:1 stoichiometry of the receptor to G protein in pertussis toxin treated cells allows for exact measure of receptor and thus G protein expression level and so the ability of an agonist to effect GTPase activity in the fused G protein. These data can be used to measure the rate of hydrolysis of GTP to GDP as affected by an agonist.

It has been shown previously that using fusion proteins between the 5-HT_{1A} receptor and G_α protein subunits can lead to an increase in the intrinsic constitutive activity of the receptor (Kellett *et al.*, 1999). These effects were most pronounced when a residue in the C terminal tail of the G_α protein involved in the coupling of receptor to G protein, Cys^{351}, was mutated to Ile^{351}. Using these properties of the fusion proteins provides a larger window of activity to screen for potential inverse agonist. However, the effect of some ligands still remains small.

The action of RGS proteins is as GAPs (GTPase activating proteins) for G_i and G_q subunits of G proteins (Dohlman and Thorner 1997; Koelle 1997; Berman and Gilman 1998). Their presence at an activated, or GTP bound G protein, results in an increase in GTPase activity, more rapid conversion of GTP to GDP and thus inactivation of the receptor generated signal. They do not affect the affinity of the G protein for GTP, only increase the rate at which it is converted to GDP. Taking advantage of this quality, RGS proteins were found to increase not only agonist stimulated but also the basal GTPase activity of the 5-HT_{1A} receptor fused to both

$G_{i1\alpha}C^{351}I$ and $G_{o1\alpha}C^{351}I$ proteins (Figure 6.1). These results demonstrated that the maximum concentration of RGS1 produced approximately a 150% increase in basal GTPase activity at the $G_{i1\alpha}C^{351}I$ fusions and a 400% increase in GTPase activity at the $G_{o1\alpha}C^{351}I$ fusion protein. Having shown a small degree of inverse activity at the 5-HT_{1A} receptor-G protein fusions in the absence of RGS1, compounds such as chlorpromazine, butaclamol and thioridazine showed a much greater level of inverse agonist activity following the increase in basal GTPase activity stimulated by the RGS protein.

In the absence of RGS1, a maximal concentration of 5-HT produces less than a two fold increase in high affinity GTPase activity in membranes expressing either the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ or 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion proteins (Figures 6.2 and 6.3). RGS1 protein produces significant increases in maximal 5-HT stimulated GTPase activity following activation of both constructs with over a three fold increase in GTPase activity for the $G_{i1\alpha}$ fusion protein over basal in the presence of RGS protein and over a two fold increase for the $G_{o1\alpha}$ protein. These effects occurred without producing a significant change in the EC_{50} value of 5-HT at the $G_{i1\alpha}$ fusion protein (Table 6.1). There was a significant effect on the EC_{50} value for the $G_{o1\alpha}$ fusion protein, 158+/-13nM in the absence of RGS1 protein and 429+/-37nM in the presence (Table 6.1). This may be due to inaccurate estimation of the EC_{50} value in the absence of RGS1 protein where a shallow Hill slope was recorded (0.56). The pK_i values from 5-HT displacement of [^{3}H]-WAY100635 binding were similar for both constructs (Table 6.2).

The 5-HT_{1A} receptor selective ligand WAY100635 has previously been reported as having antagonist properties at the 5-HT_{1A} receptor (Routledge, 1996). However, both in the absence and presence of RGS protein WAY100635 exhibited partial agonist activity at the 5-HT_{1A} receptor $G_{i1\alpha}$ and $G_{o1\alpha}$ Ile^{351} constructs (Figure 6.4 and 6.5). In the absence of RGS1 protein partial agonist effect was difficult to quantify with less than a 1.5 fold increase in basal GTPase activity was recorded. In the presence of RGS1 protein WAY100635 produced a 1.5 to 2 fold increase in high affinity GTPase activity in membranes expressing the $G_{i1\alpha}$ or $G_{o1\alpha}$ fusion proteins. This compares to a 3 fold and 2 to 3 fold increase produced by 5-HT at the same constructs respectively. No significant differences were found between the EC_{50} values in the absence or presence of RGS1 protein for either construct (Table 6.1),

with WAY100635 exhibiting the greatest affinity for the 5-HT_{1A} receptor-G protein fusions with predicted pK_i values of 8.92 and 9.05 for the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins respectively (Table 6.2). These results are supported by the finding that RGS1 protein did not have any effect on the absolute ability of [^{3}H]-WAY100635 to bind specifically to membranes expressing either 5-HT_{1A} receptor-G protein fusion (Figure 6.6).

Spiperone has been previously demonstrated to exhibit inverse agonist properties at the 5-HT_{1A} receptor fused to either WT $G_{i1\alpha}$ or the mutant $G_{i1\alpha}C^{351}I$ (Figure 6.7) proteins (Kellett *et al.*, 1999). This ligand also demonstrates inverse agonist properties in membranes expressing 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion protein (Figure 6.8) but these effects are difficult to quantitate. Spiperone produces a 50% reduction in basal GTPase activity in membranes expressing the $G_{i1\alpha}$ fusion protein in the absence of RGS1 protein with an EC_{50} of 67+/-4.3nM. The increase in basal GTPase activity in the presence of RGS1 protein makes these effects easier to quantify with out producing a significant change in EC_{50} (46+/-6.8nM). The 4 fold increase in basal GTPase activity produced by RGS1 protein in membranes expressing the 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion allows for accurate quantitation of the inverse agonist effects of spiperone. The difference in the EC_{50} for spiperone in the absence (285+/-42nM) and presence of RGS1 protein (32+/-13nM) is most likely due to an inaccurate estimation in the absence of RGS1 (Table 6.1). Spiperone displays similar ability to displace [^{3}H]-WAY100635 binding at the 5-HT_{1A} receptor fused to either the $G_{i1\alpha}$ or $G_{o1\alpha}$ mutant proteins with estimated pK_i values of 6.41 and 6.77 respectively (Table 6.2). Importantly, RGS1 protein does not have any effect on the ability of spiperone to displace the radioligand in binding studies (Figure 6.9).

The effects of spiperone on high affinity GTPase activity were further investigated over a range of GTP concentrations (Figures 6.10 and 6.11). In the absence of receptor activation RGS1 protein produced increases in both the V_{max} and K_m for GTP over basal (Table 6.3). Spiperone alone produced a decrease in V_{max}, but did not effect the K_m for GTP for membranes expressing the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins respectively. In the presence of RGS1 spiperone reduced the V_{max} and the K_m for GTP for membranes expressing the $G_{i1\alpha}$ fusion protein levels that were not significantly different from those in the absence of RGS1 protein. For membranes expressing the $G_{o1\alpha}$ fusion protein, spiperone in the presence of RGS1 protein

reduced the V_{max} and K_m for GTP to levels similar to the basal conditions for the membranes.

Of the other five compounds screened using this assay, four demonstrated inverse agonist activity: methiothepine (Figures 6.12 and 6.13), chlorpromazine (Figures 6.14 and 6.15), butaclamol (Figures 6.16 and 6.17) and thioridazine (Figures 6.18 and 6.19). Consistent with the results for spiperone, the inverse agonist effects of these compounds were difficult to quantify in the absence of RGS1 protein, particularly for the $G_{o1\alpha}$ fusion protein. In the presence of RGS1 protein though, the elevation in basal GTPase activity for membranes expressing both the $G_{i1\alpha}$ and $G_{o1\alpha}$ constructs allowed for accurate quantitation of the EC_{50} values for the inverse agonists (Table 6.2). The EC_{50} values at both the $G_{i1\alpha}$ and $G_{o1\alpha}$ protein fusions followed the same pattern with methiothepine > spiperone > butaclamol > thioridazine > chlorpromazine.

The only compound that did not demonstrate inverse agonist activity at concentrations that were relevant to receptor occupancy levels was haloperidol (Figures 6.20 and 6.21). In the absence of RGS1 protein haloperidol did not have any effect on levels of GTPase activity for membranes expressing either the $G_{i1\alpha}$ or $G_{o1\alpha}$ fusion proteins. In the presence of RGS1 protein at concentrations above 1μM there appeared to be a trend for inhibition of GTPase activity, but given the ligand concentrations these effects are most likely to be non-specific. It was clear that the lack of effect on GTPase activity by haloperidol was not due to a lack of binding as it displaced specific [^{3}H]-WAY100635 binding to membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins (Figures 6.20 and 6.21) with pK_i values of 6.49 and 6.33 respectively.

Comparison of the pEC_{50} values and the pK_i estimates for each of the compounds indicates a number of discrepancies. The pEC_{50} values from experiments using membranes expressing the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion in the presence of RGS1 protein, which would provide more accurate estimations, are not consistent with the pK_i estimations from competition bindings with [^{3}H]-WAY100635 for spiperone where a log order difference was recorded (pEC_{50} of 7.34 and pK_i of 6.41). The results for the $G_{o1\alpha}$ fusion protein show similar inconsistencies for spiperone, methiothepine and butaclamol. Further investigations could be carried out to

determine if the differences between these results are due to inaccurate estimations from either the radioligand binding assays or the GTPase assays.

A further inconsistency in the data is the difference in pK_i values for methiothepine and butaclamol between 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ proteins. The affinity of these ligands for 5-HT_{1A} receptor binding should not be effected by the G protein fused to the C terminal tail of the receptor and further studies would have to be undertaken to determine the accuracy of the pK_i estimations.

These results indicate that the use of RGS1 protein in the high affinity GTPase assay for the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ and 5-$HT_{1A}/G_{o1\alpha}C^{351}I$ fusion proteins, contributes significantly in the production of a robust and accurate screen for ligand characterisation.

Chapter 7

Conclusions

7 Conclusions

Following its discovery in 1947, and its subsequent implication in a variety of disease states, serotonin became an intensively investigated neurotransmitter. This lead to the discovery and characterisation of the 14 known 5-HT receptor subtypes, all of which are GPCRs apart from 5-HT_3, a ligand gated cation channel. These receptors have be divided into seven families, 5-HT_{1-7}, based on sequence homology and pharmacological characteristics with the GPCR members of the family generally coupling either negatively or positively to adenylyl cyclase through the $G_{i\alpha}$ and $G_{s\alpha}$ family of G proteins. The 5-HT_2 receptor family signals through the $G_{q/11\alpha}$ subtypes.

Probably the most studied 5-HT receptor is the 5-HT_{1A} (Pedigo *et al.*, 1981), in part due to the publication of its entire amino acid sequence by Kobilka *et al.* in 1987 (modified by Chanda *et al.*, 1993), but also due to the wide availability of the selective agonist 8-OH-DPAT (Gozlan *et al.*, 1983; Hjorth *et al.*, 1982) and more recently the selective antagonist WAY100635 (Routledge 1996). The 5-HT_{1A} receptor is negatively coupled to adenylyl cyclase *via* $G_{i/o}$ proteins and activates GIRK channels through the subsequent release of the $G_{\beta\gamma}$ dimer. The receptor is found centrally at high levels in the hippocampus, lateral septum, cortical areas and the mesencephalic raphe nuclei (Barnes and Sharp 1999), where it is present on serotonergic neurons both presynaptically, functioning as a classical autoreceptor, and postsynaptically where activation leads to neuronal hyperpolarisation by GIRK channel activation. The activation of presynaptic 5-HT_{1A} receptors is thought to be involved in the regulation of anxiety and depression and the activation of postsynaptic receptors has been indicated in 5-HT stimulated increases in noradrenaline and ACTH release. Both pre- and postsynaptic 5-HT_{1A} receptor activation has been indicated in the hypothermic response in rats (Barnes and Sharp 1999).

Despite the implicated roles of the postsynaptic 5-HT_{1A} receptor, the presynaptic autoreceptor has been the focus of drug research. The presynaptic receptor is already the target of a number of therapeutic regimens, directly with drugs such as buspirone and gespirone in the treatment of anxiety (Tunnicliff 1991; Barret and Vanover 1993), and indirectly by the SSRI fluoxetine (Prozac) in the treatment of depression (Artigas *et al.*, 1994, 1996).

Given that the 5-HT_{1A} receptor has also been indicated in schizophrenia, immune responses and neurogenesis, a complete understanding of the interaction between the 5-HT_{1A} receptor, and both its ligands and cognate G proteins would be of great assistance in the improvement of existing and development of new therapies.

The work described in this thesis examined the interaction of the 5-HT_{1A} receptor with both $G_{i\alpha}$ and $G_{o\alpha}$ proteins using receptor-G protein fusions. First described by Bertin *et al.*(1994), these constructs were used to investigate the interactions between the two fused proteins and the effects on ligand binding and signal generation. The actions of a class of proteins known as regulators of G protein signalling (RGS) proteins, that act as GTPase activating proteins (GAPs) for both the $G_{i\alpha}$ and $G_{o\alpha}$ proteins utilised in these studies, were also investigated. A number of different assay methods were utilised to this end, including an intact cell adenylyl cyclase, ligand binding, and GTPase assays. Throughout the work, pertussis toxin resistant G protein fusions were utilised to allow accurate measures of the G protein coupling and activation by using pertussis toxin to ADP ribosylate and uncouple the endogenous pool of inhibitory G protein. The overall findings and the implications of these on therapeutic approaches to disease states will be discussed in tandem with the future work that could be done to progress these studies further.

A number of different conclusions can be drawn from the results from the intact cell cyclase assay in the first chapter. Firstly, in support of the findings of Bahia et al. (1998) and Kellett *et al.* (1999), fusion proteins between the 5-HT_{1A} receptor and both WT and $G_{i1\alpha}$ proteins with Ile^{351} and Gly^{351} mutations indicated that the greater the hydrophobicity of the residue at position 351 in the $G_{i1\alpha}$ protein, the higher the affinity of the receptor for the G protein. This is reflected by the potency of 5-HT to stimulate inhibition of forskolin stimulated cAMP production via the 5-HT_{1A} receptor being greatest with the $Cys^{351}Ile$ mutation followed by the WT fusion and lastly by the $Cys^{351}Gly$ mutant, an order which is mirrored by a decrease in hydrophobicity. The results for the $G_{o1\alpha}$ fusion proteins in this assay were not statistically different from one another so do not support this hypothesis. This may be due to the large error in the results for these constructs, or may be a reflection of Cys^{351} not playing as significant a role for $G_{o1\alpha}$ binding to the intracellular loops of the 5-HT_{1A} receptor as for the binding of the $G_{i1\alpha}$ protein. This latter suggestion could be supported by the inability of pertussis toxin to completely abolish agonist

mediated inhibition of cAMP production in cells stably expressing the 5-$HT_{1A}/G_{o1\alpha}$ WT fusion protein and might be evident in the radioligand binding results that will be discussed later.

Comparison of the results of the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins demonstrated an obvious trend towards the $G_{i1\alpha}$ fusion proteins having a greater potency to inhibit adenylyl cyclase activity. The EC_{50} value for the 5-$HT_{1A}/G_{i1\alpha}C^{351}I$ fusion was significantly less than that of the $G_{o1\alpha}C^{351}I$ fusion protein. This result is supported by numerous studies in different expression systems documented in the literature where the 5-HT_{1A} receptor showed a higher affinity for coupling to $G_{i\alpha}$ proteins over $G_{o\alpha}$ proteins (Clawges *et al.*, 1997; Bertin *et al.*, 1992; Garnovskaya *et al.*, 1997; Raymond *et al.*, 1993).

Another pattern that emerged in the intact cell cyclase assay was for steeper Hill slope values for inhibition of cAMP production with more hydrophobic residues at position 351 on the $G_{i1\alpha}$ proteins. These data may be a reflection of the greater intrinsic constitutive activity in the fusion protein with a more hydrophobic residue at position 351, i.e. Ile^{351} being more hydrophobic and having greater constitutive activity than WT Cys^{351}, than the Gly^{351} mutant and suggest some form of positive cooperativity in the constitutively active fusion proteins.

Two final issues were addressed in Chapter 3. Firstly, inhibition of adenylyl cyclase activity following agonist stimulation of 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ was not affected by co-expression of transducin α protein. It was hypothesised that if the inhibition of adenylyl cyclase following $G_{o1\alpha}$ protein activation had been mediated by the $G_{\beta\gamma}$ dimer, co-expression of an excess of G protein α subunits, in this case, transducin α, would bind to any $G_{\beta\gamma}$ subunits that were released following $G_{o1\alpha}$ activation and prevent inhibition of cAMP production. No firm conclusion can be drawn from these experiments as the expression of transducin α was not demonstrated by Western blot analysis, although the expression of the 5-$HT_{1A}/G_{o1\alpha}C^{351}G$ which resulted in the inhibition of cAMP production indicates that efficient transient transfection did occur. The experiments would have to be repeated and expression of both receptor and G protein confirmed.

Secondly, at concentrations of 5-HT greater that 1μM, stimulation of cAMP production can be measured. It was previously suggested that this effect was due to coupling of the 5-HT_{1A} receptor to G_s at high concentrations of 5-HT (Malmberg and

Strange 2000). This seems unlikely because the same effect can be recorded in untransfected HEK293 cells where no specific binding of a 5-HT_{1A} selective radioligand can be measured. Malmberg and Strange (2000) found that 8-OH-DPAT did not stimulate adenylyl cyclase activity. As this ligand also binds the G_s linked 5-HT_7 receptor, this receptor can be excluded. The 5-HT_{5A} receptor that is G_s linked has been shown to inhibit cAMP production in a pertussis toxin dependent manner in HEK293 cells (Francken *et al.*, 1998), leaving the possible 5-HT receptors as the 5-HT_4 or the 5-HT_6 receptors. The 5-HT_4 selective antagonists GR 113808 and SB 204070 and the 5-HT_6 selective antagonists Ro 04-6790 and Ro 63-0563 could be used to determine if either of these receptors were involved. It is possible that 5-HT is activating some other class of receptor that is coupled to stimulation of adenylyl cyclase and would have to be investigated if the 5-HT_4 and 5-HT_6 receptors were also excluded.

The binding of both agonist and antagonist radioligands in two different displacement binding assays was used to measure the affinity of the 5-HT_{1A} receptor for the different G protein fusions. Firstly, the ability of increasing concentrations of GDP or suramin to displace the binding of [^{3}H]-8-OH-DPAT to the 5-HT_{1A} receptor was examined. It is well documented that a receptor coupled to a G protein has a high degree of affinity for agonists and the uncoupled form a lower affinity (Emerit *et al.*, 1990), with antagonists binding to both coupled and uncoupled receptors equally (Kobilka 1992). Both GDP and suramin have previously been demonstrated to uncouple a G protein fused to an adenosine receptor (Waldhoer *et al.*, 1999) and produce a decrease in agonist radioligand binding. These effects are produced in different manners, with GDP decreasing the affinity of the receptor for the G protein and suramin binding to the C terminal tail of the G_α subunit and acting effectively as a G protein "antagonist", competing with the receptor for G protein binding. The results for the 5-HT_{1A} receptor and its $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins gave an indication of the affinity of the receptor for the WT and mutant G proteins. As with the intact cell cyclase assay, no difference was found for the Gly^{351} and Ile^{351} mutants either with or without pertussis toxin treatment, but binding was significantly reduced for the WT G protein fusions and the 5-HT_{1A} receptor alone. However, consistent with the intact cell cyclase results for the 5-$HT_{1A}/G_{o1\alpha}$ fusion protein, a degree of agonist binding that could be displaced by increasing concentrations of

GDP or suramin was retained following pertussis toxin treatment. This again may be an indication that although Cys^{351} plays a significant role in the binding of $G_{o1\alpha}$ to the 5-HT_{1A} receptor, restricting access to this site by ADP ribosylation does not completely abolish the receptor-G protein interaction.

It was found that higher concentrations of GDP were required to displace the binding of [^{3}H]-8-OH-DPAT to both $G_{i1\alpha}$ and $G_{o1\alpha}$ with Ile^{351} than for WT G protein, with the Gly^{351} mutants requiring the lowest concentrations of GDP to produce 50% reduction in agonist binding. The exception to these results was the lack of difference between the $G_{o1\alpha}$ WT and Ile^{351} in terms of EC_{50}. The results do indicate that the 5-HT_{1A} receptor has a significantly higher affinity for $G_{i1\alpha}$ and $G_{o1\alpha}$ with the Ile^{351} mutation than with the Gly^{351} mutation. The effect of increasing concentrations of GDP was shown to be agonist dependent, as binding of the selective 5-HT_{1A} antagonist [^{3}H]-MPPF was not effected.

These results are supported by the second series of radioligand binding assays where suramin was used to uncouple the receptor-G protein interaction. As with the GDP displacement bindings, suramin provided an indication of the affinity of the 5-HT_{1A} receptor for the $G_{i1\alpha}$ and $G_{o1\alpha}$ fusion proteins with similar results. Again, 5-HT_{1A} fused to G proteins with the Ile^{351} mutation required higher concentrations of suramin to limit the binding of [^{3}H]-8-OH-DPAT to the receptor than the WT, with the Gly^{351} mutants requiring the highest concentrations of suramin. The binding of the antagonist [^{3}H]-MPPF was not affected by suramin.

As in the intact cell cyclase assay, these results may indicate that the $G_{i1\alpha}$ protein has a higher affinity for the 5-HT_{1A} receptor than the $G_{o1\alpha}$ protein. However, comparison of the EC_{50} values for displacement of [^{3}H]-8-OH-DPAT binding did not show these differences to be statistically different.

Both of these sets of agonist displacement bindings indicate that Cys^{351} plays a significant role in the binding of the 5-HT_{1A} receptor to either $G_{i1\alpha}$ or $G_{o1\alpha}$ fusion proteins and that the hydrophobicity of the residue at position 351 can affect the affinity of this binding, with greater hyrdophobicity resulting in a higher affinity of the 5-HT_{1A} receptor for the $G_{i1\alpha}$ or $G_{o1\alpha}$ proteins. The results with the WT $G_{o1\alpha}$ fusion may however indicate that although Cys^{351} does play a significant part in receptor-G protein binding, preventing binding of this site to the 5-HT_{1A} receptor does not completely prevent the interaction. The importance of other amino acids in

the binding of the 5-HT_{1A} receptor and $G_{o1\alpha}$ proteins could be assessed by site directed mutatgenesis.

A third type of radioligand binding assay was used to examine the differences between the 5-HT_{1A}/$G_{i1\alpha}$ fusions with either Ile^{351} or Gly^{351} mutations. These assays looked at the ability of a "cold" 5-HT_{1A} selective agonist to displace [^{3}H]-MPPF antagonist binding from the two fusion proteins to give an indication of the proportion of each fusion existing in the G protein coupled state. Antagonists do not affect receptor-G protein coupling and are displaced by agonists in a biphasic manner. The first phase represents the high affinity binding of the agonist to the G protein coupled form of the receptor, and the second phase the low affinity binding of the agonist to the G protein uncoupled form of the receptor. The area under the curve for each of these phases is used to give an indication of the proportion of the receptor in either form. For the 5-HT_{1A} receptor, a larger proportion of the receptor was found to be in the G protein coupled form with Ile at position 351 than with Gly at the same position. This indicates again the higher affinity of the $G_{i1\alpha}C^{351}I$ protein for the 5-HT_{1A} receptor than the $G_{i1\alpha}C^{351}G$ protein and explains the greater degree of intrinsic constitutive activity measured in the GTPase assay for the former fusion when compared to both the Gly^{351} mutant and the WT G protein.

The high affinity GTPase activity of the different 5-HT_{1A}-G protein Gly^{351} and Ile^{351} fusions were assessed in the presence of a range of concentrations of purified RGS1 and RGS16 at basal levels and following maximal 5-HT stimulation and produced some interesting results. Both RGS1 and RGS16 produced concentration dependent increases in GTPase activity for each of the constructs following 5-HT stimulation, but also did so for both the $G_{i1\alpha}$ and $G_{o1\alpha}$ proteins with the Ile^{351} mutation in the absence of agonist stimulation. This latter effect was as a result of the higher intrinsic level of constitutive activity following the Ile^{351} mutation in the $G_{o1\alpha}$ and $G_{i1\alpha}$ proteins. The effect of the RGS protein could be measured both in terms of an increase in V_{max}, the predicted maximum turnover of GTP to GDP, and K_m, the affinity for nucleotide. The latter effect is due to an increase in the rate of GTP hydrolysis (K_{cat}) and not any effect on the exchange of GDP for GTP or vice versa (K_{on} and K_{off}).

The use of pertussis toxin resistant receptor-G protein fusions has two major benefits. Firstly, it allows pertussis toxin treatment of the cells stably expressing the

constructs prior to harvesting, abolishing potential coupling to the endogenous pool of G proteins. Secondly, radioligand saturation analysis of receptor expression indicates both the expression level of the receptor and due to the 1:1 stoichiometry of the receptor to G protein, also the level of G protein expression. These two characteristics allow for the accurate determination of GTP turnover to GDP per G protein per unit time and pharmacological analysis of these results.

The turnover of GTP to GDP for each construct was plotted against RGS protein concentration and revealed that the RGS proteins did not have a significant effect on basal GTPase activity for the Gly^{351} mutants. A significant increase in activity was produced for the Ile^{351} mutants, a characteristic of the intrinsic constitutive activity that the Ile^{351} mutation confers on the 5-HT_{1A}-G protein fusion. The effects of 5-HT on GTPase turnover were also plotted against RGS concentration. 5-HT significantly increased the RGS protein effects on GTPase activity for all four constructs, with the effects of RGS1 and RGS16 not being significantly different from one another in terms of turnover.

In order to allow direct comparison of the effects of RGS protein on agonist stimulated GTPase activity, the basal turnover at each protein concentration was subtracted from the corresponding concentration following 5-HT stimulation. In the absence of agonist, both RGS1 and RGS16 proteins produced greater increases in GTPase activity at the $G_{o1\alpha}$ fusion proteins than the $G_{i1\alpha}$ fusion proteins. This could indicate either a higher level of intrinsic GTPase activity in the $G_{o1\alpha}$ proteins, or that the RGS proteins have a higher affinity for $G_{o1\alpha}$ proteins. In the presence of 5-HT (100μM), maximal concentrations of RGS protein produced greater increases in GTPase activity at the $G_{o1\alpha}$ fusions than at the $G_{i1\alpha}$ fusions, although due to the large error in the results the effects were not significant. Overall these results could indicate either that the $G_{o1\alpha}$ proteins have a greater intrinsic GTPase activity in the absence or presence of agonist, or that the $G_{o1\alpha}$ proteins have a higher affinity for RGS protein binding. The second possibility could be investigated using site directed mutagenesis of residues in the G protein α subunit that could be important in RGS protein binding. Thr^{182} is conserved in the G_i family of α subunits and is know to be a site of interaction between the G_α-GTP and RGS proteins (Burchett 2000). Gly^{183} has also been demonstrated to be required for high affinity G_α-RGS protein

interaction (Burchett 2000). Mutation of these and other residues may give an indication of the differing affinities of RGS proteins for G_α subunits.

A question arose following analysis of the results from the GTPase assays: why was no effect of endogenous RGS protein detected? The addition of purified RGS protein produces an increase in the K_m for GTP in the presence of agonist stimulation, but no equivalent increase in the K_m for GTP is recorded following agonist treatment alone, indicating that there is no significant endogenous RGS protein activity. Two approaches were used to try and reconstitute endogenous RGS protein effect, both of which investigated the possible presence of RGS protein only in the cytoplasm of HEK293 cells. A few RGS proteins are palmitoylated, e.g. RGS16, but many are predicted to be cytoplasmic proteins that are recruited to the membrane in response to G protein activation (Hepler 1999). The first experiment examined this possible recruitment of RGS proteins and by treating cells with 5-HT prior to harvesting and membrane preparation, it was hoped that a tighter association with the membrane could be induced. The second experiment examined the cytosolic cell fractions that are normally discarded during membrane preparation for RGS protein activity. Neither assay produced any RGS protein effect at either basal or following 5-HT stimulation of GTPase activity at a single concentration of GTP so full saturation analysis was not carried out. It is possible that RGS protein activity may be lost as a result of the membrane preparation and cannot be reconstituted. To investigate if RGS proteins are actually expressed in HEK293 cells QrtPCR was carried out using Taqman analysis.

The expression of a number of RGS proteins was investigated following the design of primers and probes. This initial step produced problems and it was not actually possible to design primers for many of the RGS proteins predicted to be either ubiquitously expressed or found in the human kidney as the RGS proteins were very GC rich. Primers and probes were successfully designed for RGS2, RGS3, RGS4, RGS9 and GRK2. The expression of RGS2, 3 and 4 was found in HEK293 cells at levels that would normally be predicted to be low, but this conclusion can not be strongly suggested due to problems with the assay. It thus appears that some RGS proteins are expressed, but at low levels, a finding that could explain why no endogenous effect could be detected in the GTPase assay.

The use of receptor-G protein fusions in screening assays for ligand activity has been suggested previously (Guo et al., 2001; Wurch and Pauwels, 2001) as the

data generated, especially from high affinity GTPase assays using pertussis toxin resistant G proteins, gives a very accurate pharmacological comparison of activity. The intrinsic constitutive activity of Ile^{351} $G_{i1\alpha}$ fusion proteins allowed for the comparison of both agonist and inverse agonist activity under the same assay conditions. Coupled with radioligand binding measurements of ligand affinity, the effect of maximal concentrations of RGS1 and RGS16 on the basal activity of the 5-HT_{1A}/$G_{i1\alpha}C^{351}I$ and 5-HT_{1A}/$G_{o1\alpha}C^{351}I$ fusion proteins increased the window that would allow discrimination between agonist, antagonist and inverse agonist activity.

Using this, a number of ligands that previously showed agonist, inverse agonist or antagonist activity at the 5-HT_{1A} receptor were compared in the presence and absence of 1μM RGS1 using the $G_{i1\alpha}$ and $G_{o1\alpha}$ Ile^{351} constructs over a range of concentrations using the high affinity GTPase assay. When accurate determination of an EC_{50} value could be made, no effect was seen on this in the presence of RGS protein, but a large increase in GTPase activity produced full concentration response curves that accurate pharmacological values could be generated from. The 5-HT_{1A} selective ligand WAY100635 has previously been reported to act as a neutral antagonist at the receptor with some studies on the structurally related WAY100135 producing partial agonist effects under some assay conditions (Davidson *et al.*, 1997; Schoeffter *et al.*, 1997). In the high affinity GTPase assay WAY100635 was found to act as a weak partial agonist, producing an increase in GTPase activity in the absence of RGS1 and a significant increase in the presence of RGS1.

The inverse agonist spiperone was also assayed at a maximum effective concentration (100μM) for effects on high affinity GTPase activity over a range of GTP concentrations in the absence and presence of RGS1 (1μM) at both the 5-HT_{1A} $G_{i1\alpha}$ and $G_{o1\alpha}$ $C^{351}I$ mutants. In both cases spiperone decreases the V_{max} at either basal or enhanced in the presence of RGS1 protein, and prevented the increase in K_m that the RGS protein produced at basal activity. These data indicate that the intrinsic constitutive activity induced in the 5-HT_{1A}/$G_{o1\alpha}$ and $G_{i1\alpha}$ $C^{351}I$ fusion proteins coupled with the increases in basal GTPase activity produced by the addition of RGS1 protein produce a robust and effective assay for screening ligands for agonist/inverse agonist and antagonist activity under the same conditions.

The importance of knowing accurately the class of ligand that a drug falls into is illustrated well by the 5-HT_{1A} receptor. The SSRI fluoxetine (Prozac) is used

in the treatment of depression, but its mechanism of action is far from clear. It does not produce any effects for a number of weeks following administration suggesting that its mechanism is not as simple of inhibition of the uptake of 5-HT (Artigas *et al.*, 1996). What is clear is that it reduces the levels of 5-HT found in the synaptic clefts of 5-HT neurons in the dorsal raphe nucleus, and this effect is prevented by pretreatment with pertussis toxin which uncouples presynaptic somatodendritic 5-HT_{1A} autoreceptors from GIRK channels. The activation of presynaptic 5-HT_{1A} receptors leads to a reduced firing activity in these 5-HT neurons and thus a decreased release of 5-HT itself into the synaptic cleft. The time delay of weeks in this mechanism of action may suggest that some long term alteration of 5-HT_{1A} receptor expression occurs presynaptically. However in contrast to this effect, SSRIs cause an increase in extracellular 5-HT in the median raphe nuclei. Both of these areas contain high levels of 5-HT transporters, but the dorsal raphe nuclei contain a much higher level of presynaptic 5-HT_{1A} receptors, which by the mechanism of action described above would reduce the release of 5-HT.

The co-administration of 5-HT_{1A} antagonists potentiates the antidepressant effects of SSRIs (Artigas *et al.*, 1994, 1996), an effect that would seem contradictory to the suggested mechanism of SSRI action. The co-administration leads to a larger increase in extracellular 5-HT in the striatum and frontal cortex, areas innervated by the dorsal raphe nucleus, than in the dorsal hippocampus, that is largely innervated by the median raphe nucleus. Differences in the pharmacology of the pre and postsynaptic 5-HT_{1A} receptors are well documented. For example, spiperone blocks the effect of microiontophoretically applied 5-HT in the raphe, but not in the hippocampus, and the antagonist BMY7378 is effective in the hippocampus but not the raphe. As yet no molecular differences between the pre and postsynaptic 5-HT_{1A} receptors have been shown. Another pharmacological difference is that the 5-HT_{1A} antagonist pindolol has been shown to be selective for the presynaptic somatodendritic 5-HT_{1A} autoreceptors and prevents the reduction in firing activity of 5-HT neurons induced by SSRIs.

This again demonstrates the important role that the 5-HT_{1A} receptor plays in the treatment of depression, but also the importance of developing ligands that have well characterised effects at the 5-HT_{1A} receptor. It is known that agonists at this receptor lead to downregulation with time and that inverse agonists have the opposite effect. If the effects of co-administration of SSRIs and 5-HT_{1A} antagonists rely on

alterations in the level of receptor expression, as the delay of weeks in the effectiveness of these drugs might suggest, then it must be clearly defined if a ligand has either agonist/inverse agonist or antagonist activity at the 5-HT_{1A} receptor before its therapeutic use.

The assays utilised in the various sections of this thesis would be of great benefit in the future characterisation of any such drugs.

Chapter 8

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Chapter 9

Appendix

9 Appendix

9.1 B_{max} calculation

Data from saturation binding assays were analysed using Graphpad Prism™. Nonspecific binding of the radioligand was determined by the addition of 100μM 5-HT and fitted as a straight line through the origin. This was subtracted from the total radioligand binding and the resulting specific binding in cpm plotted against the radioligand concentration. The data were fitted using a one site nonlinear regression binding hyperbola from which B_{max} and K_d were calculated.

9.2 K_i calculation

Calculation of K_i uses a derivation of the Cheng-Prusoff equation (Cheng and Prusoff 1973):

$$K_i = IC_{50}/(1+L/K_d)$$

Where L is the ligand concentration, K_d is the equilibrium dissociation constant, and the IC_{50} is the concentration of inhibitor required to inhibit half the specific binding.

9.3 GTPase Eadie Hofstee calculation

This analysis of high affinity GTPase activity data allows the calculation of both the V_{max} and the K_m for the GTPase reaction. It requires the assay of GTPase activity over a range of GTP concentrations (25nM – 3000nM approximately). The data were analysed using a Microsoft Excel spreadsheet (Figure 9.1).

The treatment column defines the concentration of GTP in each triplicate reaction. The first triplicate reaction receives only GTP from the [^{32}P]-GTP in the reaction

Figure 9.1 Example of Microsoft Excel spreadsheet used for high affinity GTPase calculation

Data from Eadie Hofstee high affinity GTPase assays were used to calculate both the V_{max} for GTP hydrolysis and K_m for GTP. The spreadsheet and the calculations involved are explained in section 9.3.

Treatment	CPM	Mean	SD	Specific CPM	Specific SD	Corr CPM	pmols	V	V/S
15.29	2369 2926 2667	2654	278.73	2608.00	278.77	2608.00	0.03	2.2109	0.1446
High GTP	51 45 42	46.00	4.58						
40.29	1931 1828 1678	1812.333333	127.23	1766.33	127.31	4854.39	0.06	3.9457	0.0979
55.29	2106 2027 2114	2082.333333	48.09	2036.33	48.30	7363.56	0.09	6.2424	0.1129
115.29	1723 1589 1699	1670.33	71.45	1624.33	71.60	12247.83	0.16	10.3831	0.0901
265.29	1032 920 853	935	90.44	889.00	90.55	15424.64	0.20	13.0762	0.0493
515.29	602 573 634	603.00	30.51	557.00	30.85	18771.52	0.24	15.9135	0.0309
765.29	576 539 532	549.00	23.64	503.00	24.08	25175.99	0.32	21.3429	0.0279
1015.29	402 476 430	436.00	37.36	390.00	37.64	25896.87	0.33	21.9540	0.0216
2015.29	284 243 220	249.00	32.42	203.00	32.74	26756.30	0.34	22.6826	0.0113
3015.29	182 166 160	169.33	11.37	123.33	12.26	24322.16	0.31	20.6191	0.0068

mixture. This varies and is defined by the half-life of ^{32}P of 14 days and is calculated for each experiment. The second treatment receives a saturating concentration of GTP that defines non-specific GTP hydrolysis. Further treatments range from the addition of 25nM to 3000nM "cold" GTP.

The CPM values from each reaction are meaned and the standard deviation calculated. The non-specific GTPase activity defined by the "High GTP" treatment are subtracted from the mean of each triplicate sample identifying the Specific CPM and the standard deviation for these mean results.

The next step corrects for the ratio between the [^{32}P]-GTP and the "cold" GTP:

Specific CPM x (total [GTP](nM)/[^{32}P-GTP](nM)) = corrected CPM

Followed by calculating the concentration of GTP hydrolysed:

Corrected CPM/CPM per pmol = pmols

The V (rate/velocity) of the reaction was calculated as follows:

(1000/2.5µg protein) x (1/20minutes) x (1000/300µl) = V

Where 2.5µg of protein was added to each reaction that was incubated for 20 minutes, from which a final volume of 300µl was counted on the Topcount.

In order to calculate the K_m using an Eadie Hofstee plot, V/S was calculated. This is simply V divided by the total GTP concentration in the reaction.